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Novel Roles for Matrix Metalloproteinases in Cell-Matrix Interactions

Anthea Jane Messent BSc.

A thesis submitted to the Open University
for the
degree of Doctor of Philosophy in cell biology.

December 1997

Department of Cell and Molecular Biology,
Strangeways Research Laboratory,
Worts Causeway,
Cambridge.

Date of award: 26th May 1998

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Acknowledgements

I would like to thank the following people, without the support of whom these studies would not have been possible. A special thanks to Richard Simpson for all his support and help. Thanks to all my colleagues and friends at Strangeways Research Laboratory, particularly Vera Knäuper for giving me her collagenase-3, and Heather Stanton and Sue Cowell for answering all my stupid questions. Finally, I would like to thank my supervisors, Jelena Gavrilovic and Gillian Murphy for all their support and guidance throughout these studies.

These studies were supported by a grant from the Cancer Research Campaign, UK.

ABSTRACT

The aim of this study was to elucidate the effect of type I collagen degradation by a physiological enzyme on cell-collagen interactions. Type I collagen is degraded *in vivo* by collagenases, which are members of the matrix metalloproteinase family of enzymes. The ability of HT1080 cells, which express the major cell-surface collagen receptor $\alpha_2\beta_1$ integrin, to attach to and invade through collagenase-cleaved collagen was investigated in cell attachment and invasion assays in comparison with the intact molecule.

Binding studies of purified, platelet $\alpha_2\beta_1$ integrin, and of ligand-binding A domain of this integrin to native collagen and to purified 3/4 and 1/4 fragments of collagen, generated by collagenase-3 revealed the necessity for retention of the triple-helical conformation of the collagen fragments for $\alpha_2\beta_1$ integrin binding. HT1080 cell attachment to type I collagen was $\alpha_2\beta_1$ integrin-mediated, and collagenase-cleavage of type I collagen perturbed cell attachment via this integrin at physiological temperature. This is likely to be due to melting of the collagen fragments, destabilizing of the triple-helical conformation of type I collagen and the resultant loss of $\alpha_2\beta_1$ integrin binding sites on the collagen molecules. However, cell attachment to the native collagen fragments was still possible at room temperature, indicating that collagenase does not destroy $\alpha_2\beta_1$ integrin binding sites on type I collagen, and that attachment can occur below physiological temperature.

HT1080 cell invasion assays through equivalent molar concentrations of native type I collagen and the 3/4 and 1/4 fragments of collagen revealed that HT1080 cell invasion through both collagen fragments was significantly greater relative to cell invasion through native collagen. These results may reflect the decreased adhesion to heat-unwound collagen fragments seen in attachment assays.

Collagenase degradation of type I collagen significantly alters $\alpha_2\beta_1$ integrin-mediated cell interactions with collagen, and also results in increased cell invasion through the degraded substrate. Matrix metalloproteinases may thus have an important role in cell-collagen interactions during tumour invasion and metastasis, where upregulation of matrix metalloproteinase synthesis by tumour and stromal cells is often observed.

Abbreviations

ADAM	A Disintegrin And Metalloproteinase
APMA	4-aminophenylmercuric acetate
bFGF	basic fibroblast growth factor
BCA	Bicinchoninic Acid
BSA	bovine serum albumin
CL-1	collagenase-1
CL-2	collagenase-2
CL-3	collagenase-3
CNBr	cyanogen bromide
DMEM	Dulbecco's modified Eagle's medium
ECL	Enhanced Chemiluminescence
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
ELISA	Enzyme-Linked Immunosorbent Assay
FAK	focal adhesion kinase
FCS	foetal calf serum
FN	fibronectin
GLA	gelatinase A
GLB	gelatinase B
ICAM	intercellular adhesion molecule
MMP	matrix metalloproteinase
MT-1 MMP	membrane type matrix metalloproteinase-1
MT-2 MMP	membrane type matrix metalloproteinase-2
MT-3 MMP	membrane type matrix metalloproteinase-3
MT-4 MMP	membrane type matrix metalloproteinase-4

LN	laminin
OG	n-octyl β-D-glucopyranoside
PBS	phosphate buffered saline
PDGFβ	platelet-derived growth factor β
SL-1	stromelysin-1
SL-2	stromelysin-2
SL-3	stromelysin-3
TBS	Tris buffered saline
TGF-β	transforming growth factor β
TIMP-1	tissue inhibitor of metalloproteinases-1
TIMP-2	tissue inhibitor of metalloproteinases-2
TIMP-3	tissue inhibitor of metalloproteinases-3
TIMP-4	tissue inhibitor of metalloproteinases-4
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VN	vitronectin
FITC	fluorescein isothiocyanate

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Chapter 1

Introduction

(i) Structure of the Extracellular Matrix

Extracellular matrix proteins such as the laminins, nidogen (entactin), type IV collagen, osteonectin (SPARC, BM-40) and heparan sulphate proteoglycans are primarily secreted by epithelial cells and assimilate *in vivo* into a complex, insoluble, macromolecular meshwork structure called the basement membrane (BM; reviewed by Mosher et al., 1992; Yurchenco and O'Rear, 1993; Timpl and Brown, 1994). The connective tissue stroma, which underlies the basement membrane in many tissues is primarily composed of a latticework of type I collagen fibres and fibronectin, secreted by connective tissue cells. Small proteoglycans such as decorin are also present in the stroma, in association with collagen fibrils (reviewed by Scott, 1995). The exact mechanism by which the basement membrane becomes linked to the underlying connective tissue is not known, however, mouse mammary epithelial cells have been demonstrated to produce heparan sulphate proteoglycans which bind to type I collagen and become stably associated with collagen fibrils (Koda and Bernfield, 1984).

(ii) Some Principal Extracellular Matrix Molecules

(a) The Laminins

The first laminin was isolated from the Engelbreth-Holm-Swarm (EHS) tumour. Since then many other tissue-specific laminin isoforms have been identified, reviewed by Paulsson (1993). Laminins are characterized by their domain structure; one heavy (A or α) chain and two light (B1 or β , and B2 or γ) chains. These are generally arranged into cross-shaped molecules, in which the N-terminal regions of the three domains form

the short arms of the cross and the bulk of the three domains associate together into the long arm of the cross (reviewed by Engel, 1993; Timpl and Brown, 1994). Laminins self-assemble into large networks which interact with many other constituents of the ECM, for instance nidogen, thereby providing a structural basis for basement membrane assembly.

(b) Type IV Collagen

There are six known varieties of type IV collagen α -chains, $\alpha 1$ - $\alpha 6$. Monomers of type IV collagen are composed of a tissue-specific combination of three α -chains wound into a triple helix. The α -chains possess multiple interruptions in their triple helix conformation, resulting in highly flexible fibrils. Monomers of type IV collagen aggregate into fibrils which assimilate into an insoluble meshwork. The C-terminal domain of the type IV collagen chains which is globular in structure (the NC1 domain), and the N-terminal domain (7S domain) which is rich in cysteines and lysines, are thought to play a major role in type IV collagen molecule polymerisation. Lateral association of type IV collagen monomers via their triple-helical domains is also possible (reviewed by Kühn, 1995). Cross linking of collagen IV fibrils contributes to formation of a stable, nonreversible scaffolding for basement membranes.

(c) Proteoglycans

Proteoglycans are proteins which have one or more attached glycosaminoglycan side chains which are covalently bound to the core protein. The glycosaminoglycans side chains are composed of either chondroitin sulphate, dermatan sulphate, keratan sulphate or heparan sulphate. Many proteoglycans, such as aggrecan and betaglycan, carry more than one type of side chain (reviewed by Hardingham and Fosang, 1992). Chondroitin sulphate proteoglycans found in mammals (such as aggrecan, decorin and biglycan) are most abundant in cartilage and are essential for distributing load in weight-bearing

joints. Of the heparan sulphate proteoglycans, perlecan is the most predominant and well characterized (reviewed by Noonan and Hassell, 1993) and is the principal proteoglycan found in basement membranes.

Proteoglycans are found both in the extracellular matrix, and on the cell-surface (discussed later in this chapter). The role of the extracellular matrix proteoglycans is still under investigation, however, several aspects of their function has been elucidated. The principal physical properties of the negatively charged glycosaminoglycan side chains is that they act as water attractants, and also exclude macromolecules from the matrix whilst remaining permeable to smaller soluble molecules. Thus ECM proteoglycans keep the matrix hydrated and may concentrate extracellular macromolecules or act as a molecular "sieve". Proteoglycans bind to each other and to other ECM components and cell-surface molecules such as type IV collagen and heparan sulphate. The structural role of proteoglycans in basement membrane is thought to be that of linking and filling molecules, binding the laminin and type IV collagen networks together and interweaving the gaps in the meshwork structure. Proteoglycans such as decorin may also have a role in ECM organization, by binding to collagens type I and II and modulating collagen fibril formation (reviewed by Hardingham and Fosang, 1992).

(d) Type I Collagen

Type I collagen is a member of the fibrillar collagen family, and is one of the most abundant components of the ECM in many tissues. It is particularly abundant in skin, tendons, ligaments, uterus, large blood vessels and bone, which all require high tensile strength without loss of flexibility. Fibrillar collagens (types I, II and III) consist of three polypeptide chains (α -chains) that are coiled into a semirigid helical structure. Type I collagen is composed of two $\alpha 1(1)$ chains and one $\alpha 2(1)$ chain (fig.1.1). These chains are very similar but are the products of two separate genes located on the human chromosomes 17 and 7 respectively (reviewed by Chu and Prockop, 1993). The α

chains each contain just over 1,000 amino acids and have molecular weights of approximately 95,000 kDa. Thirty three percent of the amino acid residues of fibrillar collagens (including type I collagen) are glycines, and these are arranged at every third residue to give rise to a polymer of tripeptide units, -Gly-X-Y-. This repeating tripeptide sequence is critical for triple-helix formation, and imparts structural integrity to collagen fibrils. Some flexibility is conferred by the imino acid composition of the helix tripeptides, Gly-Pro-Hyp being much more rigid than Gly-Pro-Y or Gly-X-Hyp. The C-terminal end of the triple-helical domain of the $\alpha 1(I)$ and $\alpha 2(I)$ chains contains a fivefold repeat of the Gly-Pro-Hyp triplet which forms a very stable region during triple-helix formation at this potentially weak point of the molecule. The triple-helical conformation occurs throughout 95% of the length of the rod-like monomer, leaving a few N-terminal and C-terminal residues (telopeptides) in a less rigid conformation.

Type I procollagen α -chains are translated with an N-terminal signal peptide sequence. This signal peptide is proteolytically removed when the pro α -chains reach the endoplasmic reticulum (ER). Following entry into the ER, type I collagen pro α -chains undergo extensive post-translational modification, including hydroxylation, glycosylation and triple-helix assembly. Proline and lysine residues in the -Y- position of the -Gly-X-Y- triplets are hydroxylated by specific hydroxylases (reviewed by Kieley et al., 1993).

All three hydroxylating enzymes require the collagen peptide chains to be in the non-helical conformation, therefore, hydroxylation must occur before triple-helix formation of the pro α -chains. Hydroxylation of these specific proline residues directly contributes to folding and stabilization of the procollagen α -chains into triple-helical molecules. Hydroxylated lysine (hydroxylysine) residues are further modified in the ER by glycosylation. This is achieved by a glucosyl- and a galactosyl-transferase enzyme, and the resulting glycosylation of procollagen α -chains is thought to facilitate fibril organization. Association of the procollagen α -chains occurs initially via residues within the C-terminal region of the peptides and is stabilized by disulphide bond

formation. The initial interaction of pro α -chains allows propagation of triple helix folding, prior to procollagen secretion via the Golgi apparatus into the extracellular space.

Specific extracellular metalloproteinases (procollagen N-proteinase and procollagen C-proteinase) process procollagen to the mature form by removal of the globular N and C-terminal propeptide domains (reviewed by Kielty et al., 1993). The resulting molecules are primarily triple-helical, rod shaped proteins which spontaneously aggregate into homo- and heterotypic fibrils (reviewed by Kühn, 1987; Kielty et al., 1993; Brodsky and Ramshaw, 1997). These fibrils become cross-linked from within and between the collagen molecules, which serves to stabilize and strengthen the collagen lattice. The cross-linked collagen fibrils are arranged into quarter-staggered arrays and organised according to their tissue type (e.g. parallel bundles in tendon; partially oriented coarse network in skin).

(iii) The ECM as an Effector of Cell Activity

The BM has been found to be of great importance in maintenance of tissue structure by providing a scaffold resistant to mechanical forces and physically separating the tissue compartments, but other biological activities such as sequestration and inhibition of growth factors like TGF- β by decorin have been reported (reviewed by Ruoslahti and Yamaguchi, 1991). Other growth factors such as basic FGF are bound by heparan sulphate-containing ECM proteoglycans and are brought into close association with cells, and allowing bFGF binding to its cell surface receptor (Ullrich and Schlessinger, 1990). The significance of ECM-bound growth factor influence on cell activity has been reviewed by Taipale and Keski-Oja (1997), and these are likely have a major influence on cell behaviour during events such as neoplastic growth of primary tumours and tumour metastasis (discussed later in this chapter).

Culture of cells on ECM components, such as type I collagen gels, have been shown to directly regulate activation of matrix-degrading enzymes such as gelatinase A (Azzam and Thompson, 1992). Culture of cells in three-dimensional type I collagen gels has also been shown to induce fibroblast (Langholz et al., 1995) and osteosarcoma cell expression of collagenase-1 (Riikonen et al., 1995). Other authors have reported that culture of fibroblasts in type I collagen gels upregulated activation of endogenous fibroblast gelatinase A, relative to gelatinase A produced by fibroblasts cultured on thin collagen films (Seltzer et al., 1994). These enzymes are responsible for ECM remodeling and may profoundly alter cell function (discussed in more detail later in this chapter). Cell contact with the ECM regulates other aspects of cell behaviour, including prevention of apoptosis (Boudreau et al., 1996) or smooth muscle cell proliferation (Koyama et al., 1996), cell attachment to the ECM (Shrestha et al., 1996; Wu et al., 1996), mammary carcinoma cell response to bFGF (Elliott et al., 1992) and tumour cell migration and invasion (reviewed by Ruoslahti, 1992; Chintala et al., 1996). The pathway by which ECM-mediated regulation of cell activity is achieved is discussed in more detail later in this chapter.

Proteolytic fragments of basement membrane proteins have also been shown to effect cell interactions with the extracellular matrix. Type I collagen fragments produced by collagenase degradation of type I collagen have been shown to be chemotactic for human dermal fibroblasts (Albini and Adelmann-Grill, 1985). Gelatinase-A cleavage of laminin-5 was shown to generate an altered form of laminin-5 which induced migration of breast epithelial cells (Giannelli et al., 1997). Angiogenesis, the process by which new blood vessels are formed by endothelial cells lining existing vasculature, is influenced both at the cell-adhesion and cell-proliferation stages by cleaved ECM components (reviewed by Sage, 1997). In addition, the cell differentiation and migration-stimulatory effects of native plasma fibronectin have been shown to be exposed only after incubation of the fibronectin with matrix metalloproteinases (Fukai et al., 1995).

The breadth of influence of the ECM on cell behaviour is still not fully understood and therefore under intense examination, however, it is clear from the above results that cell interactions with the ECM have the potential to modulate most aspects of cell activity. Regulation and/or maintenance of these interactions are crucial to cell function and are discussed below.

(iv) Cell Surface Receptors for Extracellular Matrix

Several classes of cell-surface receptors for ECM are expressed by cells. These include glycoproteins such as the integrin family of receptors (discussed later in this chapter), and glycoproteins such as the heparan sulphate proteoglycans, CD44 glycoproteins and the syndecans. CD44 was originally identified as the lymphocyte homing receptor which bound to endothelial cell addressin, but has since been found to be expressed by many cell types in both low and high molecular weight forms. The differences in CD44 composition appear to be tissue or cell-type specific. The high molecular weight forms, such as found on epithelial cells, bear glycosaminoglycan side chains such as heparan sulphate and chondroitin sulphate. The low molecular weight forms, the predominant form found on lymphocytes, are not proteoglycans. CD44 has been shown to bind to fibrillar collagen, fibronectin, tenascin and hyaluronan in some cell types (reviewed by Hardingham and Fosang, 1992; Wright et al., 1992).

Syndecans are a family of integral membrane, heparan sulphate-carrying proteoglycans bind to interstitial matrix proteins such as fibrillar collagen, fibronectin and tenascin via their heparan sulphate and chondroitin sulphate side chains (reviewed by Hardingham and Fosang, 1992; Rapraeger, 1993; Elenius and Jalkanen, 1994). Ligand binding by syndecans is specific, as syndecans do not bind to laminin-1 or vitronectin despite their heparin binding domains. It has also been proposed that syndecans may interact with integrins to mediate cell adhesion (reviewed by Elenius and Jalkanen, 1994), thus co-

ordination of adhesion receptor expression may be necessary to bring about attachment of some cell types to the ECM.

(v) Structure and Function of the Integrins

(a) Integrin Structure

A principal group of cell surface receptors for ECM proteins are the integrins. Integrins are heterodimeric glycoproteins composed of non-covalently associated α and β subunits (reviewed by Hynes, 1992; Kühn and Eble, 1994; Newham and Humphries, 1996) (fig.1.2). At least fourteen α subunits and 8 β subunits have been identified to date, although the total number of possible subunit combinations is limited by the fact that not every α subunit is able to associate with every β subunit (Table 1.1). Often the α subunits are only able to complex with a single, common β subunit, although the α_v integrin subunit is a notable exception to this case.

Each integrin subunit is anchored into the cell membrane by a single hydrophobic transmembrane domain. Most integrins (apart from the β_4 integrin subunit) have short cytoplasmic tails of less than 50 amino acids, with no evidence of intrinsic tyrosine kinase activity. Electron microscopy of several integrins show a globular head comprising parts of both subunits, supported by two stalks which extend into the plasma membrane (Carrell et al., 1985; Nermut et al., 1988). The N-terminal half of all α subunits contain a seven-fold repeat structure, parts of which are thought to be involved in binding of divalent cations, which are essential to the function of some integrins as cell adhesion receptors (Staatz et al., 1989; Kirchhofer et al., 1991; Grzesiak et al., 1992). The ligand binding domain of the α subunits is also thought to be contained within the seven-fold repeats, but this site is unlikely to be at the same location as the cation binding site (Masumoto and Hemler, 1993). A common feature of the β subunits is a four-fold repeat of a disulphide-bonded, cysteine-rich domain

which is thought to be involved in ligand binding (for a review of integrin structure, see Loftus et al., 1994; Takada et al., 1997).

Some α subunits (α_1 , α_2 , α_D , α_E , α_L , α_M and α_X) also have an extra domain of approximately 180 amino acids, known as the I-(inserted) domain, between repeats II and III of the seven-fold repeat structure. These domains are homologous to the A-domain modules of von Willebrand factor (Preti et al., 1987) which mediates collagen binding, and A-domain-like sequences found in type VI collagen, complement factor B and cartilage matrix protein (Colombatti et al., 1993). The inserted regions of the integrins are therefore usually referred to as A-domains rather than I-domains. The A-domain of the α_2 integrin subunit has been demonstrated to be the ligand-binding region of the molecule (Kamata and Takada, 1994; Tuckwell et al, 1995) and it is likely that the A-domains of the other integrins will also be involved in ligand binding. Lee et al. (1995) postulated that the β_3 integrin subunit may also contain an A-domain module and evidence for putative A-domains in other β subunits have recently been published (Tuckwell et al, 1995; Tozer et al, 1996; Tuckwell and Humphries, 1997).

Binding of divalent cations such as Mg^{2+} , Mn^{2+} and Ca^{2+} induces reversible conformational changes in the integrin heterodimer extracellular domains. Binding of the stimulatory cations (Mg^{2+} or Mn^{2+}) is essential for inducing a conformational change which promotes ligand binding (integrin-activation) (reviewed by Humphries, 1996). Cation dependence was demonstrated by addition of the divalent metal ion-chelator ethylenediaminetetraacetic acid (EDTA), which abolishes integrin-mediated cell adhesion (Santoro, 1986; Diamond and Springer, 1994). Integrin activation may also induce changes in the conformation of the cytoplasmic domains of the integrin subunits, potentially leading to alterations in the signals transduced by the active versus inactive integrin (see below). Binding of Ca^{2+} generally induces an inactive conformational state in most integrins, (when Ca^{2+} is in the mM concentration range), which does not permit ligand binding to the integrin (Humphries, 1996).

(b) Integrin Function; Integrins as Signal-transducing Molecules

In addition to being receptors for matrix proteins, integrins have second, more significant role, as signal transducing molecules (reviewed by Clark and Brugge, 1995; Richardson and Parsons, 1995; Dedhar and Hannigan, 1996; Yamada, 1997a and references therein). Cell regulation of integrin-mediated interaction with the ECM is usually as a direct consequence of transduction of information about the extracellular environment either via integrins themselves or other cell-surface receptors (reviewed by Juliano and Haskill, 1993; Roskelley and Bissell, 1995; Humphries, 1996). Signaling via integrins occurs in two directions; inside-out signaling which regulates the affinity and conformation of the receptors, and outside-in signaling which is triggered by ligand binding of the receptors and can lead to a multitude of responses including modulation of integrin-mediated adhesion. Signaling is mediated by the cytoplasmic domains of the integrins, particularly the β subunits (reviewed by Sastry and Horwitz, 1993; Laflamme et al., 1997; O'Toole, 1997), but since the integrins possess no intrinsic kinase activity of their own they must interact with other proteins to activate signals.

The precise mechanism by which signals are transmitted following ligand binding is not yet fully established, although recent papers have shed much light onto this process. Ligand-bound integrins are recruited into specialized structures called focal adhesions, which also involve proteins of the cytoskeleton and signaling proteins such as focal adhesion kinase (FAK; reviewed by Guan, 1997). Marcantonio and David (1997) proposed a mechanism by which the short cytoplasmic tail of the integrin β subunits might become involved with other proteins. Briefly, they postulate that the α subunit maintains integrin signaling latency when the receptor is ligand-unoccupied by prevention of the formation of α -helical structures within the integrin β subunit cytoplasmic domains. Ligand binding is thought to alter the relationship of the cytoplasmic domains which leads to formation of the α -helices needed for interaction

with signal transducing proteins. Most β integrin subunits have tyrosine residues with conserved motifs which may be phosphorylated, leading to signal transduction via classical signaling pathways such as Ras/MAP kinase cascades, although other mechanisms may be possible (Laflamme et al., 1997; O'Toole, 1997). Ligand binding by some integrins, such as $\alpha_2\beta_1$ integrin, has also been shown to trigger release of intracellular calcium and bring about calcium-mediated cell signaling (reviewed by Sjaastad and Nelson, 1997).

The mechanisms governing inside-out signaling or integrin activation are less well understood, although some progress has recently been made in this area (reviewed by Ginsberg et al., 1992; Dedhar and Hannigan, 1996). Activation of several integrins has been shown to be mediated by novel cytoplasmic proteins binding to specific β integrin subunits. Examples of this include activation of lymphocyte β_2 integrin by cytohesin-1 (Kolanus et al., 1996) and activation of platelet β_3 integrin by endonexin (Shattil et al., 1995). Another novel integrin binding protein, named integrin linked kinase (ILK), has been found to bind β_1 , β_2 and β_3 integrin cytoplasmic domains (Hannigan et al., 1996). Integrin activation has also been shown to be regulated by divalent cations such as Mg^{2+} and Ca^{2+} . Mg^{2+} and Mn^{2+} have been postulated to bind to the integrin at "Ligand Competent Sites" and may thereby regulate the rate of ligand association. Ca^{2+} , on the other hand, may bind the integrin at "Allosteric Inhibitory Sites" which prevent ligand binding and therefore regulates the rate of ligand dissociation (Smith, 1997).

(vi) Regulation of Integrin Activity

Cells are able to regulate integrin-mediated interactions with the ECM at multiple levels. Firstly, several integrins have been shown to have alternatively spliced regions in their cytoplasmic tails which may account for the difference in specificity of a given integrin from cell type to cell type, or within the same cell at different stages in development

(Kirchhofer et al., 1990; Fornaro and Languino, 1997). Secondly, the specificity and affinity of a given integrin on a cell are not always constant (reviewed by Diamond and Springer, 1994). A well documented example of this has been reported for the platelet $\alpha_{IIb}\beta_3$ integrin on the surface of resting platelets, which is unable to bind soluble fibrinogen until the platelet is activated by agonists such as collagen or thrombin (Phillips et al., 1991). Platelet activation induces a conformational change in the $\alpha_{IIb}\beta_3$ integrin which permits binding to soluble fibrinogen. Cells are also able to regulate adhesion and migration on the ECM by modulation of their integrin repertoire (Skinner et al., 1994; Beauvais et al., 1995). An example of this phenomenon is the loss of $\alpha_v\beta_3$ and gain of $\alpha_5\beta_1$ integrin in a highly metastatic melanoma cell line (C8161) in comparison to a moderately invasive melanoma cell line (A375M) (Seftor et al., 1993). Modulation of integrin expression and activation states can have profound effects on processes as diverse as keratinocyte differentiation (Adams and Watt, 1990), tumour cell proliferation, cell survival (Giancotti, 1997; Meredith and Schwartz, 1997) and metastasis (reviewed by Varner and Cheresch, 1996).

Studies using mice in which integrin subunits have been "knocked out" have shown that complete ablation of the β_1 subunit causes early embryonic death (Fässler and Meyer, 1995a). In contrast, mice which carry a homozygous null mutation for the α_1 integrin and α_3 integrin subunits develop to adulthood and are fertile. Ablation of the α_1 integrin subunit confers no apparent phenotype, suggesting other integrins are able to compensate to a large extent for the loss of $\alpha_1\beta_1$ integrin (Gardner et al., 1996). However, loss of the α_3 integrin disrupts development and maintenance of the epidermal basement membrane and leads to blistering at the dermal-epidermal junction of skin (DiPersio et al., 1997). Therefore, expression of some integrins are essential for normal development, although loss of single integrins such as $\alpha_1\beta_1$ integrin may be compensated for due to the overlapping ligand specificities of many integrins.

(vii) Cell Interactions With Type I Collagen

The principal type I collagen receptors on cells are the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (Gulberg et al., 1992; Santoro and Zutter, 1995). Some groups have reported $\alpha_3\beta_1$ integrin binding to type I collagen (Grenz et al., 1993; Yamamoto and Yamamoto, 1994), but this is probably restricted to a few select cell types. $\alpha_2\beta_1$ integrin is expressed by many cell types and is thought to exist in at least three different functional activation states (Chan and Hemler, 1993), all of which are functionally Mg^{2+}/Mn^{2+} -dependent. It is thought that the activation state of the integrin may be responsible for the differences in ligand binding specificity, as $\alpha_2\beta_1$ integrin has been found to be a receptor for laminin as well as collagens on some cell types (Elices and Hemler, 1989; Kirchhofer et al., 1990). This integrin can be activated from the resting state (unable to bind collagens) into the activated state by activating anti- β_1 integrin antibodies, which bind the β_1 subunit at a location distinct from the ligand binding site and induce a conformational change into the activated (collagen-binding) integrin conformation (Arroyo et al., 1992; Humphries, 1996; Wilkins et al., 1996). The ability of inactive $\alpha_2\beta_1$ integrin to undergo activation *in vitro* suggests that cells may also be able to manipulate $\alpha_2\beta_1$ integrin activity *in vivo*. Unlike many of the other integrins, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin binding to ligands is an RGD-independent process. Cardarelli et al. (1992) reported that a cyclic RGD (single letter amino acid code)-containing peptide inhibited $\alpha_2\beta_1$ integrin binding to type I collagen, but Eble et al. (1993) found that these peptides had no effect on $\alpha_1\beta_1$ integrin binding to type I collagen. Staatz et al. (1991) reported that platelet binding to type I collagen could be inhibited by a DGEA-containing peptide, suggesting that this was the $\alpha_2\beta_1$ integrin adhesive motif on type I collagen. However, other reports have not confirmed this result (reviewed by Tuckwell and Humphries, 1996b).

Cell attachment to denatured fibrillar collagens has been postulated to be mediated by cryptic RGD sites which become exposed upon unwinding of the collagen triple helix.

$\alpha_v\beta_3$ integrin has been suggested to bind to the exposed RGD peptides (Davis, 1992; Montgomery et al., 1994), however, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin interactions are completely ablated by collagen denaturation. Cell attachment to denatured type II collagen has also been shown to be mediated by $\alpha_5\beta_1$ integrin. This rather unexpected result was explained by the collagen-binding ability of fibronectin, which was shown to form a bridge between cells and denatured type II collagen (Tuckwell et al. 1994). This result is potentially important for maintenance of cell attachment to a substratum which has been subject to proteolytic remodeling.

$\alpha_2\beta_1$ integrin has been found to bind to collagens I-VI and XI, although its interactions with type I collagen are the best understood. Several groups have identified regions of the type I collagen molecule thought to be involved in $\alpha_2\beta_1$ integrin binding. The most frequently reported data relates to the $\alpha_1(I)$ chain cyanogen bromide fragments numbers 3 and 7 ($\alpha_1(I)$ -CB3; Staatz et al., 1990) (fig.1.3), although other regions of the collagen molecule have been shown to support $\alpha_2\beta_1$ integrin-mediated cell adhesion too (Fitzsimmons et al., 1986; Morton et al., 1994). One study pinpointed the $\alpha_2\beta_1$ integrin recognition sequence as the tetrapeptide DGEA, which corresponds to residues 435-438 of the $\alpha_1(I)$ -CB3 fragment of type I collagen (Staatz et al., 1991), however, at present this is not conclusively defined. As has already been mentioned, the location of the collagen binding site on $\alpha_2\beta_1$ integrin is much more clearly defined, Kamata and Takada (1994) and Tuckwell et al (1995) independently reported that recombinant $\alpha_2\beta_1$ integrin A-domain bound specifically to type I collagen. A later study extended this to collagens type I, II, IV and XI but not to synthetic triple-helical collagenous peptides, suggesting that the A-domain is able to recognize sequence-specific ligands (Tuckwell et al, 1996a).

Despite the structural diversity of collagenous molecules with which $\alpha_2\beta_1$ integrin is able to interact, a common feature is the requirement for collagen triple-helical conformation in order to support $\alpha_2\beta_1$ integrin adhesion. This has been demonstrated

using non-physiological, cyanogen bromide derived peptides of collagen (Vanderberg et al., 1991; Pfaff et al., 1993; Morton et al., 1994; Ruggiero et al., 1994; Tuckwell et al., 1994). This is potentially a critical feature of $\alpha_2\beta_1$ integrin-mediated cell adhesion to collagens and laminin, as it is well documented that loss of cell contact with the ECM can lead to apoptotic cell death (reviewed by Ruoslahti and Reed, 1994; Meredith and Schwartz, 1997). Pilcher et al. (1997) showed that keratinocyte migration was maintained when cells were prevented from adhering firmly to type I collagen by denaturation of the substrate. *In vitro* studies using cells transfected with $\alpha_2\beta_1$ integrin (Keely et al., 1995) and $\alpha_7\beta_1$ integrin (Echtermeyer et al., 1996) have shown that expression of an integrin type not previously expressed by the cell type in question can dramatically increase cell motility over ECM proteins, suggesting that cells may also be able to respond to external stimuli by induction of integrins which are not normally expressed by the particular cell type. This has important implications for the processes of tumour cell transformation, invasion and metastasis, where gain or upregulation receptors such as $\alpha_2\beta_1$ integrin (Santala et al., 1994), β_1 integrins (Bartolazzi et al., 1994) and β_3 integrins (Albelda et al., 1990; Danen et al., 1995a) have been reported (reviewed by Juliano and Varnier, 1993; Tang and Honn, 1994).

A study by Keely et al. (1995) showed that expression of α_2 integrin antisense mRNA in a human breast carcinoma cell line reduced cell adhesion to types I and IV collagen, without effecting adhesion to fibronectin or laminin. Cells which exhibited intermediate levels of adhesion to collagen were found to be more motile across collagen-coated filters during haptotaxis assays. The level of motility over collagen directly corresponded with the level of antisense α_2 integrin expressed by the cells; highly expressing cells showed unchanged levels of migration, but low levels of adherence, weakly expressing cells showed increased adherence but a low level of migration. Antisense α_2 integrin expressing cells were also unable to organize into glandular epithelial-like structures when cultured in three-dimensional collagen gels, unlike the parent cell line. Therefore,

$\alpha_2\beta_1$ integrin is essential for maintenance of collagen-dependent morphogenic capacity of mammary epithelial cells.

Recent studies have highlighted the link between integrin-mediated signal transduction and signaling via classical signaling pathways. Signaling via integrins are thought to activate signaling pathways shared with mitogens such as growth factors. This can lead to convergence of adhesion-mediated and growth factor-mediated signalling pathways, transduced by proteins such as Ras, MAPK and Rho (reviewed by Dedhar and Hannigan, 1996; Giancotti, 1997). The integration of the soluble and adhesion-dependent signals has been shown to be vital for cell co-ordination of events such as cell proliferation and migration (McNamee et al., 1993; Jones et al., 1996b). Even more interestingly, Schneller et al. (1997) reported that a small, highly tyrosine-phosphorylated fraction of the PDGF β receptor and the insulin receptor co-immunoprecipitated with the $\alpha_v\beta_3$ integrin from human foreskin fibroblast cells. This supports the idea that growth factor-growth factor receptor complexes may be recruited into focal adhesion complexes within the cell membrane (Plopper et al., 1995), leading to a highly complex association of signaling proteins and activation of a single signaling cascade.

(viii) Structure and Function of the Extracellular Matrix-Degrading Matrix Metalloproteinases

Extracellular matrix remodelling is mediated by proteolytic enzymes, particularly by members of the matrix metalloproteinase (MMP) family (reviewed by Matrisian, 1992; Murphy and Reynolds, 1993; Birkedal-Hansen, 1995). MMPs can be synthesized by many cell types including connective tissue cells, haemopoetic and tumour cells. MMPs are part of the metzincin group of proteinases, which are derived from a larger family of zinc-dependent metalloproteinases. All nineteen mammalian MMPs discovered to date have a putative Zn^{2+} -binding site and are dependent on Ca^{2+} for stability, however,

they can be broadly subdivided into 5 groups based on their structure and substrate preference (Table 1.2). All MMPs carry a propeptide domain which is lost during activation, (discussed later in this chapter), and a catalytic domain which contains the active site of the enzyme. The majority of MMPs (except matrilysin) also carry a C-terminal domain which is composed of hemopexin-like repeats. The C-terminal domain is joined to the N-terminal catalytic domain by a flexible linker sequence (fig.1.4).

Collagenases are members of a very small group of physiological proteinases which are able to degrade triple-helical collagens. There are three vertebrate collagenases known at present, collagenase-1 to -3. Collagenase-1 (CL-1) was originally described by Gross and Lapiere (1962) as the enzyme responsible for tadpole tail involution, and was the first vertebrate MMP to be discovered. CL-1 is produced by many cell types including fibroblastic cells and bone cells (reviewed by Harris and Krane, 1974). Later, a second collagenase (CL-2, MMP-8), which is structurally different from CL-1, was isolated from neutrophils (Hasty et al., 1990). This collagenase has since been found to also be produced by chondrocytes in human cartilage, and to be upregulated in osteoarthritic cartilage and in response to interleukin-1 (IL-1) (Chubinskaya et al., 1996). The primary fibrillar collagen present in articular joints is type II collagen, and this is likely to be the principal substrate of CL-2 produced by chondrocytes and neutrophils in normal and arthritic joints. Interestingly, CL-2 is also able to cleave the major articular proteoglycan, aggrecan, although CL-2 is not now thought to be the primary enzyme responsible for aggrecan degradation (Arner et al., 1997). The most recently discovered collagenase, collagenase-3 (CL-3, MMP-13), was isolated from a human breast tumour library (Freije et al., 1994). Collagenase-3 has since been expressed, purified and biochemically characterized (Knäuper et al., 1996a; Knäuper et al., 1997a; Knäuper et al., 1997b).

Fibrillar collagens (collagen I, II and III) are highly resistant to proteolytic degradation due to their triple-helical structure. However, the mammalian collagenases exhibit C-

terminal domain binding to the collagen triple helix resulting in the destabilization of this region and subsequent cleavage (De Souza et al., 1996). A recent study by Lemaître (1997) showed that recombinant mouse collagenase-3 catalytic domain expressed in *Escherichia coli* was able to degrade gelatin, casein and the N-telopeptides of type I collagen, although the catalytic domain on its own was not able to cleave the triple-helical domain of collagen. The importance of the C-terminal domain of CL-3 for interaction with fibrillar collagens was demonstrated, since deletion of this region ablated triple-helicase activity and also reduced CL-3 interaction with TIMPs 1 and 3 (Knäuper et al., 1997b). These report and others confirm the necessity for the C-terminal domain of collagenases in mediating collagen cleavage (De Souza et al., 1996; Gomis-Rüth et al., 1996). The hinge region of collagenases is also important for maintenance of the stability and collagenolytic ability of collagenases (Knäuper et al., 1997a), and it has been suggested from computer modeling studies that this region may assume a collagen-like conformation (De Souza et al., 1996). The region of the collagen triple-helix surrounding the collagenase-cleavage site contains less imino acids is less stable than the rest of the triple-helix (reviewed by Gross et al., 1980; Kielty et al., 1993). Destabilization or relaxation of the triple helix allows collagenases to cleave all three chains of type I collagen at a single locus between residues 775 and 776, that is, between one Gly-Ile in each $\alpha 1$ chain and one Gly-Leu in the $\alpha 2$ chain (Highberger et al. 1979). The fragments generated are approximately 3/4 and 1/4 of the total length of the native molecule and melt below 37°C (Sakai and Gross, 1967; Stark and Kühn, 1968a). Interestingly, Krane et al. (1996) showed that rodent interstitial collagenase, which is highly homologous to human CL-3, cleaves type I collagen at two locations. As well as cleaving at the classical site within the collagen triple helix, rodent collagenase has an additional cleavage site within the aminotelopeptide region. Human collagenase-3 has also recently been demonstrated to cleave at this additional, aminotelopeptide, cleavage site (Knäuper et al., 1997b). Liu et al. used mice which expressed mutant collagen, that was resistant to collagenase degradation at the classical triple-helical site, to show that rodent collagenase cleavage of type I collagen at the

nonhelical N-telopeptide site was important for resorption of collagen during development.

Gelatinases were so called because they are very efficient at degrading denatured collagens. Native type IV collagen was also thought to be a substrate for the gelatinases but this was later proved to be due to a reducing step during the purification of type IV collagen. Neither gelatinase A (GLA) or B (GLB) have been found to cleave unreduced type IV collagen (Eble et al., 1996). The catalytic domain of gelatinases contain three repeated units which are closely related to the type II domains of fibronectin. These fibronectin-like repeats are unique to gelatinases amongst the MMPs and are responsible for mediating gelatinase binding to gelatin (Murphy et al., 1994a). *In vitro*, gelatin is generated by heat-denaturation of collagens and is usually reversible if the gelatin is allowed to slowly renature at room temperature. *In vivo*, this situation is not possible and therefore true gelatin can only be generated by collagenase degradation of collagen into the 3/4 and 1/4 fragments (an irreversible process). As already mentioned, the fragments generated by collagenase degradation of collagens are not stable at physiological temperature and probably denature into gelatin as soon as they are formed. Since these gelatinized collagens and collagenase-generated fragments of collagens are extremely susceptible to degradation by many proteinases including gelatinases, it is unlikely that gelatins are present in the extracellular environment for a significant length of time. However, this does not mean that gelatins have no physiological role to play, and the dramatic change in cell substrate from highly structured, fibrillar collagen to random coil, gelatinized alpha chains has been shown to have significant impact on cell interactions with the substrate (Tucker et al., 1990; Pilcher et al., 1997).

Stromelysin-1 and -2 (SL-1 and SL-2) have the widest substrate specificity of the MMPs identified to date (see Table 1.2). These are unusual among the soluble MMPs in that they are only activated by proteinases outside of the MMP family (e.g. plasmin,

trypsin, elastase, cathepsin G; (Nagase et al., 1990)). Stromelysin-3 (SL-3) has a more restricted substrate specificity than SL-1 and SL-2, and has been shown to be activated intracellularly prior to secretion (discussed in more detail later in this chapter).

The most recently discovered members of the MMP family are the membrane-type matrix metalloproteinases (MT-MMPs). Four MT-MMPs have been identified to date (Sato et al., 1994; Takino et al., 1995; Will and Hinzmann, 1995; Puente et al., 1996). The transmembrane domain of these MMPs localizes their activity to the pericellular environment, however, MT-1 MMP has been shown to degrade most of the ECM proteins in the test tube (Pei and Weiss, 1996). MT-1 and -2 MMPs have also been demonstrated to be involved in the activation of GLA, and this is discussed later in this chapter.

(ix) Cellular Regulation of Matrix Metalloproteinase Activity

MMP activity is regulated at three separate levels: synthesis, activation and inhibition (reviewed below).

(a) Synthesis

Transcription of many MMPs is mediated by the interaction of AP-1 and a number of other transcription factors with specific promoter sequences within the MMP genes (reviewed by Benbow and Brinckerhoff, 1997). Transcription of MMPs can be regulated by cell interactions with the ECM, for example: attachment of rabbit synovial fibroblasts to peptides derived from fibronectin induced collagenase and stromelysin expression (Werb et al., 1989), and decorin regulated fibroblast expression of collagenase when cultured on vitronectin (Huttenlocher et al., 1996). Other examples of ECM regulation of MMP synthesis include; laminin, type IV collagen, fibronectin and tenascin stimulation of mouse macrophages to express GLB (Khan and Falcone, 1997),

and induction of keratinocyte expression of collagenase-1 by cell contact with native type 1 collagen (Sudbeck et al., 1997). Interestingly, for keratinocytes, contact with type I collagen induces CL-1 expression, however, contact with laminin-1 represses CL-1 expression (Sudbeck et al., 1997). Expression of MMPs can be influenced by integrin-mediated signaling, for example, perturbation of the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins on invasive melanoma cells have been shown to upregulate to a small extent gelatinase A expression and secretion (Seftor et al., 1992; Seftor et al., 1993). MMP synthesis can also be induced by stimulation of cells with cytokines and growth factors, such as EGF (Shima et al., 1993; Zeigler et al., 1996), PDGF (Yanagi et al., 1992), TGF- β (Overall et al., 1991) and TNF- α (Jasser et al., 1994).

(b) Activation of Matrix Metalloproteinases

All the MMPs described so far, with the exception of stromelysin-3 (SL-3) and the membrane-type matrix metalloproteinases (MT-MMPs), are secreted in the latent form which requires extracellular processing to the active form (reviewed by Nagase, 1997). MMPs are multi-domain structures composed of the N-terminal pro-region which is lost during activation, followed by the Zn^{2+} - and Ca^{2+} -binding catalytic domain. The C-terminal domain is composed of a 4-bladed β -propeller which shows strong sequence similarity to the hemopexin family of proteins. The C-terminal domain is connected to the catalytic domain by a hyperflexible linker, or hinge, of variable length (fig.3; Birkedal-Hansen, 1995). The propeptide region occupies the active site cleft of the catalytic domain by ligation of an invariant Cys residue in the propeptide sequence to the zinc ion. Stepwise activation of the MMP is achieved by initial proteolytic cleavage which destabilizes the Cys- Zn^{2+} interaction, which then allows further proteolysis to the fully active form (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990). The serine proteinase, plasmin, is thought to be involved in the initial catalytic step necessary during autocatalytic activation of many of the MMPs including; stromelysin-1, and -2 (SL-1 and -2; Nagase et al., 1990), gelatinase-B (GLP-B; Ogata et al., 1992)

and collagenase-1 (CL-1; Gavrilovic and Murphy, 1989). Other active MMPs may greatly increase the activity of plasmin-activated MMPs for example, incubation of SL-1 with plasmin-activated CL-1 elicits a 10-fold increase in collagenase activity at a molar ratio of 1:100 (Murphy et al., 1987). Both fibroblast and neutrophil collagenases are superactivated by stromelysin-1, and neutrophil collagenase has also been shown to be activated by stromelysin-2 (Knäuper et al., 1996c).

Pro gelatinase A (pGLA) has been found to be activated at the surface of cells treated with concanavalin A (Con-A) (Murphy et al., 1991). A recently identified membrane-bound MMP, (MT-1 MMP; Sato et al. 1994), is thought to mediate GLA activation by partial cleavage of the pro-GLA to an intermediate form, which is then processed to the mature form by autolytic cleavage of the GLA itself (Atkinson et al., 1995). This proteolytic cascade activation mechanism has a similar function to the stepwise activation observed for other MMPs, representing multiple opportunities for regulation of MMP activity at the cell surface (reviewed by Basbaum and Werb, 1996). A further level of complexity to the GLA activation pathway is the involvement of tissue inhibitor of metalloproteinases-2 (TIMP-2). TIMP-2 has been isolated from the plasma membrane of Con-A treated HT1080 cells and was found to be in a complex with MT-1 MMP and GLA. GLA activation was enhanced by the addition of low levels of exogenous TIMP-2 but inhibited at higher doses of the TIMP (Strongin et al., 1995). TIMP-2 forms a complex with pro-GLA via the N-terminal domain of the TIMP and the C-terminal domain of pro-GLA, leaving the N-terminal domain of GLA free to interact with MT-1 MMP. The importance of the C-terminal domain of GLA during GLA activation has been demonstrated (Murphy et al., 1991; Ward et al., 1994), loss of this part of the molecule prevented pGLA binding to the cell surface and activation of the enzyme. It is possible that TIMP-2 acts as an "anchor" protein during GLA activation, binding free pGLA and holding it in position in close proximity to the cell surface, thereby enabling MT-MMP interaction with GLA to initiate activation. However, the exact role of TIMP-2 in the GLA activation mechanism has yet to be elucidated.

Interestingly, CL-3 has also been shown to be activated at the cell surface by MT-1 MMP and gelatinase A (Knäuper et al., 1996b). Similarly to gelatinase A activation, cell-surface activation of collagenase-3 is also dependent on its C-terminal domain (Knäuper et al., 1997b).

MT-1 MMP, along with SL-3, has been shown to be activated intracellularly by the Golgi-associated subtilisin-like proteinase, furin (Pei and Weiss, 1995; Pei and Weiss, 1996). Due to the transmembrane domain of the MT-MMPs, the active enzymes are anchored at the cell surface, unlike SL-3 which is fully secreted as a soluble, active enzyme. The activation mechanism of the other MT-MMPs has not yet been published, however, since these also contain the furin recognition motif it is likely that these too will be activated prior to insertion into the cell membrane.

(c) Inhibition of MMPs

Once activated, MMPs are regulated *in vivo* by specific inhibitors of MMPs, the tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs have been identified to date (Stetler-Stevenson et al., 1989; Uria et al., 1994; Leco et al., 1997) and these are often coexpressed with MMPs by cells, therefore allowing tight regulation of matrix degradation (reviewed by Edwards et al., 1996). The four TIMPs are distinct gene products and are composed of two structurally distinct domains. TIMP-1 is a 30 kDa glycoprotein which forms high-affinity, non-covalent, complexes with active MMPs in a 1:1 stoichiometry. TIMP inhibition of MMPs does not cause permanent changes in MMP conformation and therefore the interaction is in theory reversible, although the affinity of the inhibitor for the MMP is so great that very stable complexes are formed (Murphy et al., 1994b). Some specificity of TIMP interactions with MMPs is possible as it has been shown that TIMP-1 does not completely inhibit MT-1 MMP activity but that TIMP-2 does (Atkinson et al., 1995). Of interest is the fact that TIMP-1 binds to

proGLB and TIMP-2 binds to proGLA, which is of importance to the activation of the latter, as discussed above.

Both TIMP-1 and TIMP-3 expression can be induced by growth factors such as EGF and TGF- β 1, whereas TIMP-2 expression is more constitutive (reviewed by Edwards et al., 1996). However, TIMP-3 is different from all other TIMPs described to date in that subsequent to secretion, it becomes bound to the ECM. This may reflect an additional level of cell regulation of MMP activity, since restriction of TIMP-3 to an area proximal to the cell surface may allow TIMP-3 the greatest influence on pericellular ECM proteolysis. Point mutations in the TIMP-3 gene have been shown to be present in patients suffering from Sorsby's fundus dystrophy (SFD), a condition which effects the retina and leads to irreversible blindness (Weber et al., 1994). The significance of synthesis of dysfunctional TIMP-3 protein and the SFD disease is not yet known, although increased MMP activity due to TIMP-3 malfunction is unlikely since thickening and vascular penetration of Bruch's membrane is a feature of the disease.

An imbalance between MMP and TIMP production where levels of secreted MMP exceeds the available extracellular TIMP has been postulated to be the cause of many instances of pathological tissue destruction, for example during tumour cell metastasis. The current model of the role of MMPs and TIMPs in tumour growth and invasion is examined in more detail later in this chapter.

(x) The ADAMs: Cell Surface Proteins Possessing a Metalloproteinase and an Adhesion Receptor Domain

A novel family of cell surface proteins with A Disintegrin And Metalloproteinase domain, the ADAMs, have recently been described. These proteins have a unique domain organisation consisting of a proteolytic domain, an adhesion domain, a fusion domain and a signaling domain. The proteinase and cell adhesion domains of the

ADAMs share ~30% sequence homology with the snake venom metalloproteinases, which are soluble proteins, unlike the membrane-bound ADAMs. Despite all the known ADAMs possessing the same four domains, sequence analysis of the proteins suggests that not all domains in all ADAMs are functional. At least 15 vertebrate ADAMs have been identified to date, of these, 1-6 have been implicated in sperm-egg fusion during fertilization. Others have been identified on macrophages, myoblasts, brain and some tumour cells (reviewed by Wolfsberg and White, 1996).

Seven of the full-length vertebrate ADAMs contain an extended active-site sequence motif which is similar to that found in the catalytically active metalloproteinase domain of the snake venom metalloproteinases. This suggests that these ADAMs can be catalytically active in the pericellular environment and it has recently been demonstrated that a new member of the ADAM family processes the precursor of tumour necrosis factor α (TNF- α), to allow release of the mature cytokine from the cell surface (Black et al., 1997; Moss et al., 1997). Since cytokines and growth factors have such an important role during normal and neoplastic cell proliferation and motility, this finding suggests that ADAMs may be involved in mediating processes such as induction of tumour growth and metastasis.

The disintegrin domains of the ADAMs are similar to those found on snake venom proteins, which have been demonstrated to bind to the platelet $\alpha_{IIb}\beta_3$ integrin and disrupt platelet aggregation (reviewed by Wolfsberg and White, 1996). The integrin-binding region of the snake venom disintegrin is thought to be a 13-14 amino acid loop which contains an RGD sequence at the tip. The RGD-containing loop projects out from the rest of the disintegrin and probably binds to a pocket in the $\alpha_{IIb}\beta_3$ integrin heterodimer. Much less is known about the disintegrin domains of the ADAMs, however, the sequence of the predicted 14-amino acid loop is much more degenerate amongst the ADAMs than the snake venom disintegrins, which might indicate that not all ADAM disintegrins are able to bind to the integrins or other surface receptors.

The first two ADAMs identified were originally isolated from guinea pig sperm, and their roles in the reproductive process has been the subject of intense research (reviewed by Wolfsberg and White, 1996; Bigler et al., 1997). It was originally thought that the metalloproteinase domain of fertilin α (ADAM-1), might be important for spermatid cell migration through the seminiferous tubules during early spermatogenesis. However, a recent report suggesting that the human fertilin α gene is non-functional casts some doubt on the role of this ADAM during reproduction (Jury et al., 1997). The role of fertilin β (ADAM-2), which forms a heterodimer with fertilin α on guinea pig and bovine sperm, during sperm-egg interactions is better understood. The major functional domain of fertilin β is thought to be its disintegrin domain, which has been postulated to be involved in sperm-egg binding via interactions with an egg integrin.

The cytoplasmic tails of the ADAMs have been postulated to be involved in signal transduction (reviewed by Wolfsberg and White, 1996). This is due to the fact that several of the ADAM cytoplasmic domains, such as meltrin gamma, are rich in proline which may allow binding of signaling molecules (Weskamp et al., 1996). Others are rich in lysine and serine, and some have consensus sites for phosphorylation. However, the cytoplasmic domains of the ADAMs are not well conserved across mammalian species and therefore the importance of ADAMs as signal transducing molecules has yet to be elucidated.

(xi) Mechanism and Regulation of Cell Motility

Migration of cells can occur via three different processes; chemotaxis, haptotaxis and chemokinesis. Chemotaxis is the directional movement of cells towards a gradient of a specific, soluble attractant (reviewed by Albini et al., 1985). Haptotaxis is cell movement guided by an immobilized attractant, and chemokinesis is the random migration of cells caused by factors which stimulate motility.

Cell motility is achieved by a fine balance of cell attachment and detachment (reviewed by Huttenlocher et al., 1995; Opas, 1995; Adams, 1997). A well studied example of this is the "rolling mechanism" employed by leukocytes to move along the vascular endothelium (Dunon et al., 1996). Integrins have a central role in potentiation of cell motility over extracellular matrix, enabling cells to maintain simultaneous interactions with both the extracellular matrix and the actin cytoskeleton. Initiation of cell locomotion involves a directional protrusion of the leading edge of the cell to form a lamellipodium, a structure which is firmly linked to the ECM via focal adhesion complexes. These focal adhesions also serve to link the substratum and the actin cytoskeleton which is essential to provide the traction required to drag the cell in the direction of the lamellipodium. Ligand occupancy of β_1 integrins has been shown to lead to retrograde movement of the ligand-bound integrins mediated by attachment of the β_1 integrin cytoplasmic tail to the cytoskeleton, thereby generating the contractile force necessary for cell locomotion (Felsenfeld et al., 1996).

In order to permit cell movement, integrin attachments to the ECM at the rear of the cell must be released. Most integrins at the trailing edge have been shown to be recycled to the leading edge of the cell (Lawson and Maxfield, 1995; Palecek et al., 1996), however, integrins and other cells surface proteins have been found to be deposited on the ECM at the rear of the cell (Friedl et al., 1997), indicating that the recycling process is not 100% efficient.

Cell migration is regulated by a number of external factors. These include growth factors, cytokines and ECM components (reviewed by Huttenlocher et al., 1995). Anti-adhesive ECM components such as tenascin, thrombospondin and SPARC can induce cell migration by promoting detachment from the substratum and possibly by induction of MMP expression (Tremble et al., 1993; Tremble et al., 1994), leading to degradation of the ECM and consequently cell detachment. Growth factor signaling has been shown

to stimulate $\alpha_v\beta_5$ integrin-mediated carcinoma cell migration on vitronectin. However, in the absence of the growth factor stimulus, carcinoma cells migrated over collagen using $\alpha_2\beta_1$ integrin, and $\alpha_v\beta_5$ integrin was only found to be involved in carcinoma cell adhesion to vitronectin (Klemke et al., 1994).

Cell motility is also regulated by the strength of the adhesions formed by integrins to the ECM (Palecek et al., 1997). At low adhesive strength cells will not be able to generate sufficient traction to permit migration, however, at higher adhesive strength cells will not be able to relax their adhesions to permit migration, effectively trapping the cell on the substratum. Variations in the substrate concentrations and cell surface integrin expression can effect the rate of cell migration, intermediate levels of both receptor and substrate tending to lead to maximal migration (DiMilla et al., 1991). A second way in which the strength of cell attachment can be regulated is by modulation of integrin activation states. As has been previously explained, this is mediated by the cytoplasmic domains of the integrins and is effected by inside-out signaling. Thus, cells have several levels of opportunity for regulating cell motility, which is tightly controlled in normal tissue, but deregulation of which can lead to tumour cell metastasis.

(xii) The Role of MMPs in Tumour Invasion and Metastasis

Tumour transformation from the benign to the malignant state involves interactions between the primary tumour and the ECM. These involve breakdown of the basement membrane by metastatic cells derived from the primary tumour, invasion of these through the connective tissue stroma, lymph vessels or basement membranes of small blood vessels, (intravasation), and transport in the bloodstream to a distant site. Metastatic cells must then adhere to the internal surface of blood vessels and extravasate into the new site, and finally degrade the stroma at the new site to allow expansion of the metastatic lesion and infiltration of blood vessels (reviewed by Hart and Saini,

1992). All these processes require proteolysis of the ECM and tissue remodeling, which could be potentially mediated by MMPs.

MMP-mediated hydrolysis of basement membrane has been thought to be a critical step during tumour cell intravasation into the bloodstream and extravasation into a new tissue. The involvement of MMPs in tumour progression has been noted for many years, however, a clear picture of the precise role of MMPs during this process has yet to be conclusively drawn. Many studies have found upregulation of MMPs and/or downregulation of TIMPs associated with tumours or the surrounding stroma (reviewed by Stetler-Stevenson et al., 1993; MacDougall and Matrisian, 1995b; Sato and Seiki, 1996; Basset et al., 1997; Parsons et al., 1997). More direct evidence for the importance of MMP activity during metastasis has been the downregulatory effect of TIMPs and synthetic MMP inhibitors on tumour cell invasion and metastasis, both *in vitro* and *in vivo* (reviewed by Chambers and Matrisian, 1997; Docherty et al., 1997; Parsons et al., 1997).

Despite upregulation of MMP activity around invasive tumour cells, degradation of ECM must be under some degree of control, otherwise tumour cells would be unable to gain sufficient adhesion to the substratum to permit movement towards the bloodstream and target organs. This may be regulated by TIMP production by stromal cells or even the tumour cells themselves, as TIMPs have occasionally been found to be upregulated in malignant tumours (Grignon et al., 1996). Positive regulation of MMP production by stromal cells surrounding the tumour may be achieved by production of growth factors and cytokines by tumour cells. MMPs are also not the only proteinases capable of ECM degradation and it has been demonstrated that enzymes of the plasminogen-activator system cooperate with MMPs during tumour progression (reviewed by DeClerck and Laug, 1996). This is particularly significant as plasmin has already been shown to activate several MMPs *in vitro*, and this may represent a highly sensitive mechanism for regulation of MMP activity. Signal transduction via integrin receptors has also been

shown to effect MMP production in normal and tumorous cells (DeClerck and Laug, 1996), suggesting that the extracellular environment may provide another level of regulation of MMP-mediated tumour cell invasion (Sudbeck et al., 1997).

An interesting report by Koop et al. (1996) showed by using the technique of intravital videomicroscopy that normal and malignant, ras-transformed NIH 3T3 fibroblasts were equally well able to extravasate, however, the difference in normal versus transformed phenotypes was manifest during postextravasation growth. Although the MMP profile of the normal versus transformed fibroblasts was not explored in this study, MMP synthesis and activation has often found to be a feature of associated with malignant transformation. Upregulation of MMP activity in the tissue surrounding tumours was thought to be important for permission of tumour cell metastasis (reviewed by Stetler-Stevenson et al., 1993; Powell and Matrisian, 1996), although another study by Koop et al. (1994), found that overexpression of TIMP-1 in B16F10 melanoma cells had no effect on the rate of cell extravasation into chick embryo chorioallantonic membrane. However, this study showed that overexpression of TIMP-1 markedly suppressed post-extravasatory tumour growth. Therefore, MMPs may be capable of influencing neoplastic cell growth and growth of metastatic lesions (reviewed by Chambers and Matrisian, 1997). This suggests a more subtle role for MMPs during metastasis than had been previously ascribed. MMP facilitation of tumour growth may be indirect, possibly by release of sequestered growth factors from the ECM by MMP degradation and/or by degradation of the surrounding matrix to increase the space available for the tumour to occupy. MMPs have also been implicated in angiogenesis. Studies using synthetic inhibitors of MMPs have found angiogenesis to be downregulated by MMP inhibition (Galaray et al., 1994). It is possible MMPs are acting in concert with growth factors such as vascular endothelial cell growth factor (VEGF), which have an established role in angiogenesis (Kim et al., 1993).

The abundance of data concerning MMPs and tumour progression confirms their relevance to the metastatic process. New reports concerning more the subtle influence of MMPs during metastasis are now appearing in the literature, and comprehensive elucidation of the role of MMPs during tumour growth and metastasis will hopefully lead to novel tumour-suppressant drugs and more specific targets for therapeutic intervention.

(xiii) In Summary

The role of MMPs during tumour cell proliferation and metastasis remains to be clearly defined. It is apparent from previous work that MMP production and activation by tumour and stromal cells comprises a single aspect of tumourigenicity, although this area may still offer a potential target for therapeutic treatment. A more detailed knowledge of the co-ordination of cytokine signaling and ECM signaling which bring about changes in cell behaviour, such as MMP expression, is vital to the design of effective treatments. Therefore, studies which focus on a particular aspect of the proposed metastatic cascade are important for elucidating the effects of a single event, such as MMP cleavage of a substrate. It is hoped that by defining individual issues during tumour progression that a detailed understanding of the whole, complex process can be built up.

This study focuses on the role of CL-3 in regulation of $\alpha_2\beta_1$ integrin-mediated cell interactions with type I collagen. By presenting cells with either native collagen or collagenase-cleaved collagen fragments, it is possible to clearly evaluate the effects of CL-3 cleavage on cell attachment to, and invasion through, collagen-1. The majority of published data to date concerning $\alpha_2\beta_1$ integrin-mediated cell-collagen interactions, relates to cell binding to non-physiological, cyanogen bromide fragments of type I collagen, often assayed below 37°C. This study is designed to address the apparent absence of information regarding the physiological significance of collagenase cleavage

of type I collagen. Since collagenolytic cleavage of fibrillar collagens may be an instance of in vivo generation of gelatin (denatured collagen) it is important to evaluate the effects of collagenase activity on cell-collagen interactions at physiological temperature.

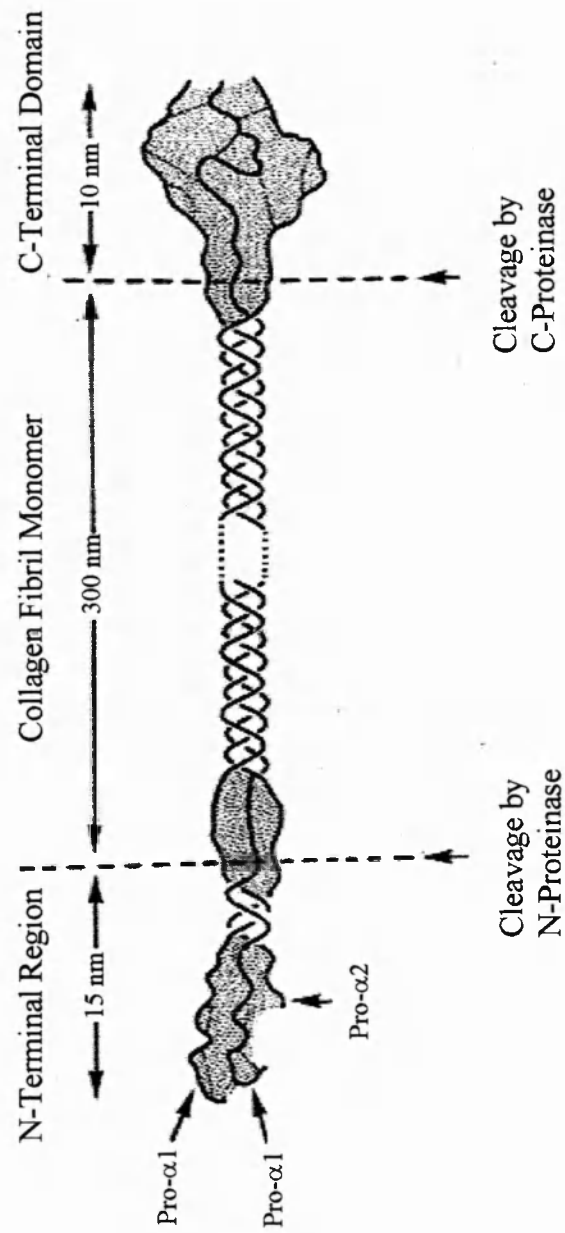


Fig.1.1: Diagrammatic representation of type I procollagen (reproduced from Jackson, 1980). The N-terminal and C-terminal propeptide domains are proteolytically removed to produce the mature type I collagen monomer.

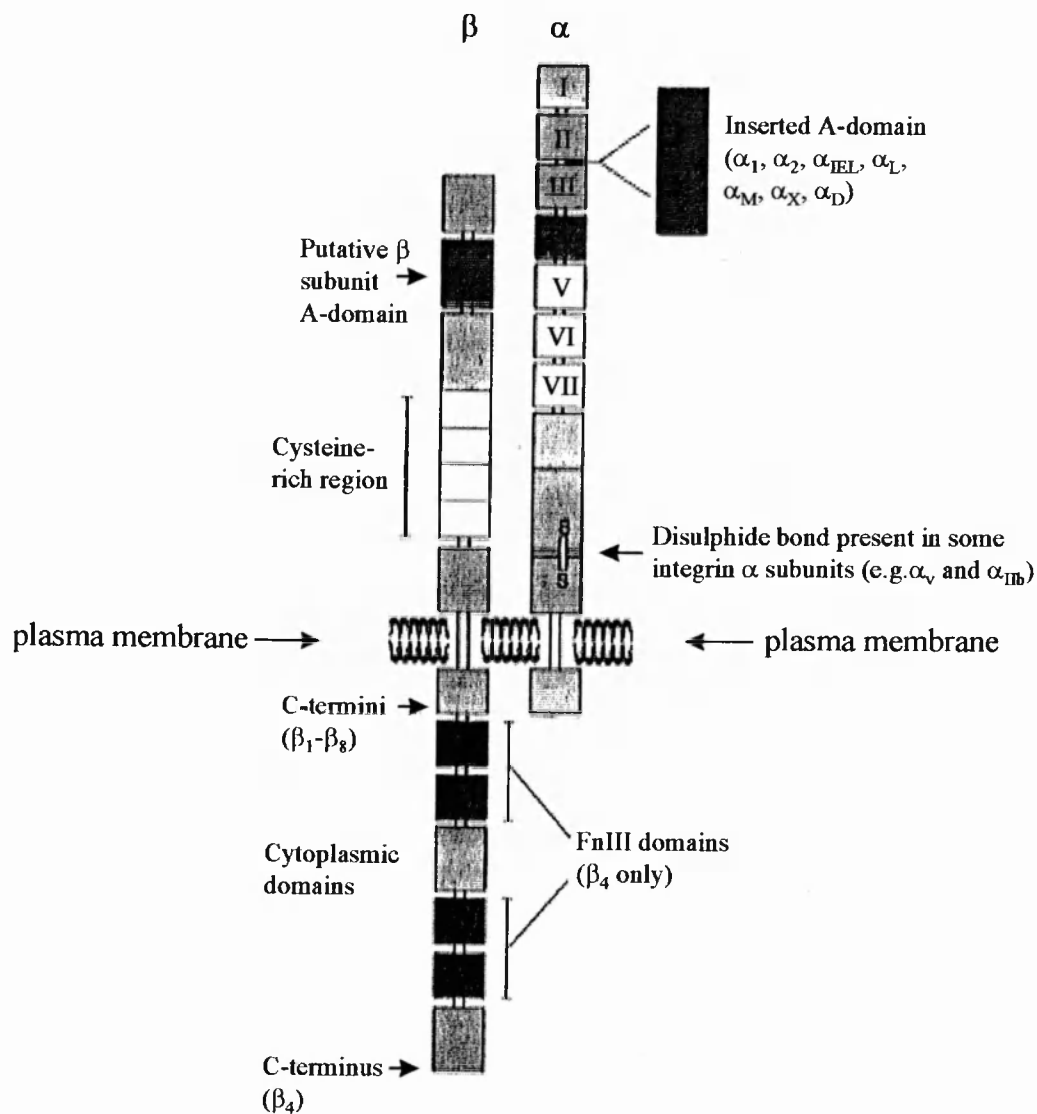
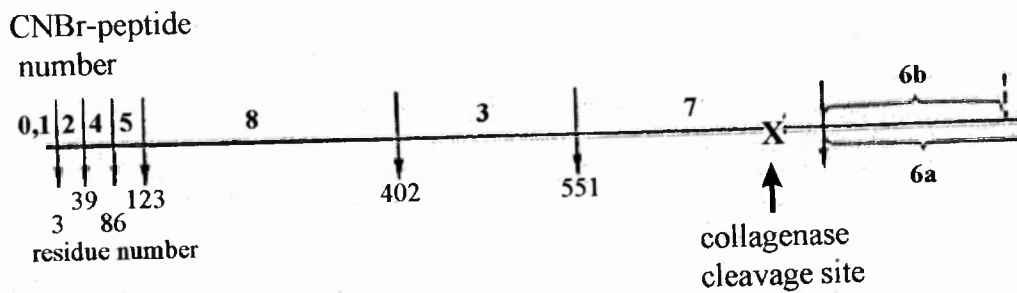
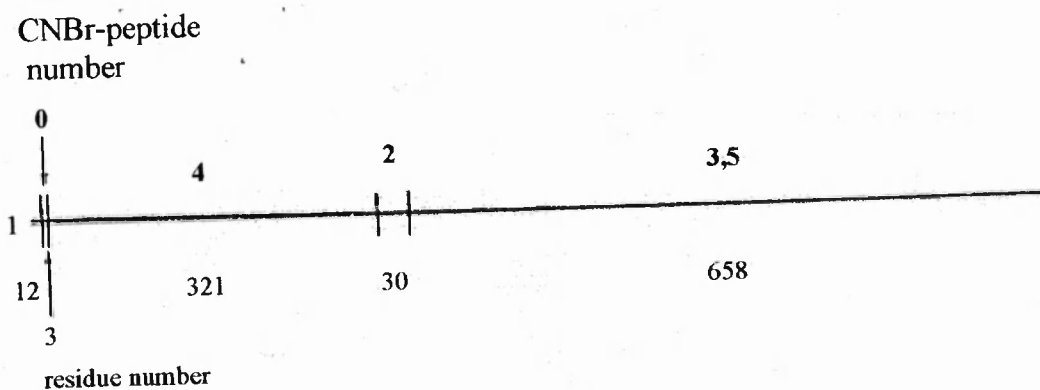


Fig.1.2: Schematic diagram of an integrin heterodimer (taken from Newham and Humphries, 1996), showing the various domains of the α and β integrin subunits. Key features such as the A-domains, the cysteine-rich region of the β subunit and the seven-fold repeated domains of the α subunit are marked. Most integrins have very short cytoplasmic tails, the exception is the β_4 subunit, which has a considerable cytoplasmic domain.



$\alpha 1(I)$ -chain



$\alpha 2(I)$ -chain

Fig.1.3: Diagrammatic representation of the type I collagen molecule. Type I collagen is composed of two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain which assimilate into a triple helix. The position of the classical mammalian collagenase cleavage site is marked, as are the positions at which cyanogen bromide fragments the collagen molecule. The CNBr peptide number is marked above the corresponding region of the collagen α chains.

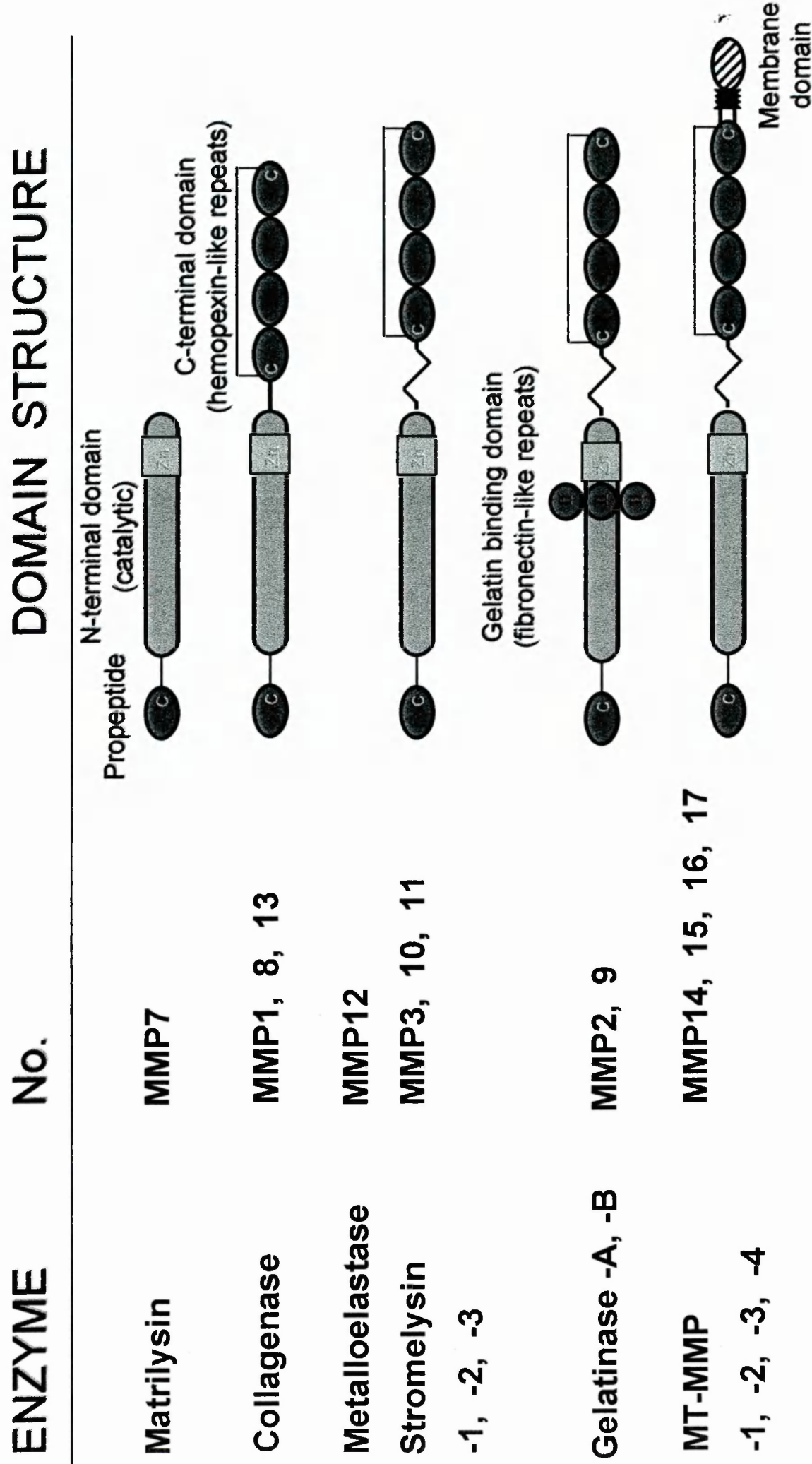


Fig.1.4: Matrix metalloproteinases can be broadly categorised by their domain structure, as shown in the diagram above.

Table 1

Subunit	Subunit	Ligands and Counter Receptors
β_1	α_1	collagens, laminin
	α_2	collagens, laminin
	α_3	fibronectin, laminin, collagens
	α_4	fibronectin, VCAM-1
	α_5	fibronectin
	α_6	laminin
	α_7	laminin
	α_8	not known
	α_v	vitronectin, fibronectin
	α_L	ICAM-1, ICAM-2
β_2	α_M	fibrinogen, factor X, ICAM-1, complement (C3b)
	α_X	fibrinogen, complement (C3b)
β_3	α_{IIb}	fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin
	α_v	vitronectin, fibronectin, fibrinogen, von Willebrand factor, thrombospondin, osteopontin, denatured collagens
β_4	α_6	laminin
β_5	α_v	vitronectin
β_6	α_v	fibronectin
β_7	α_4	fibronectin, VCAM-1
	α_{IEL}	not known
β_8	α_v	not known

ICAM: intercellular adhesion molecule; VCAM: vascular cell adhesion molecule

Table 2

Enzyme	MMP number	Substrates
<i>Collagenases</i>		
Collagenase-1	1	collagens I, II, III, VII and X, aggrecans, serpins, α_2 -macroglobulin (α_2 M), gelatins (limited)
Collagenase-2	8	collagens I, II and III, aggrecans, serpins, α_2 M, gelatins (limited)
Collagenase-3	13	collagens I, II, III, IV, IX, X and XIV, gelatins, serpins, aggrecan, fibronectin, laminin, tenascin, N-telopeptides
Collagenase-4 (Xenopus)	18	collagen I
<i>Gelatinases</i>		
Gelatinase A	2	denatured fibrillar collagens, collagens V, VII and XI, α_2 M, tenascin, elastin
Gelatinase B	9	denatured fibrillar collagens (I-III), collagens V, VII, XI and XIV, α_2 M, elastin
<i>Stromelysins</i>		
Stromelysin-1	3	aggrecan, fibronectin, nidogen, laminin, proteoglycan core protein, collagen IX, gelatins (limited), elastin (limited), nonhelical X-links of collagen IV
Stromelysin-2	10	aggrecan, fibronectin, non-helical X-links of collagen IV, proteoglycan core protein, collagen IX, laminin, gelatins (limited), elastin (limited)
stromelysin-3	11	α_1 -proteinase inhibitor, limited activity on fibronectin, laminin, collagen IV, aggrecan, gelatins
<i>Membrane-type MMPs</i>		
MT1-MMP	14	collagens I, II, III, fibronectin, laminin, vitronectin, dermatan sulphate proteoglycan, proMMP-2, proMMP-13
MT2-MMP	15	not known
MT3-MMP	16	proMMP-2
MT4-MMP	17	not known
<i>Others</i>		

Matrilysin (PUMP)	7	aggrecan, fibronectin, laminin, gelatins, non-helical X-links of collagen IV, elastin, entactin, collagen IX, proteoglycan core protein, vitronectin
Metalloelastase	12	elastin
(Unnamed)	19	not known

Chapter 2

Materials and General Methods

The majority of chemicals and reagents used in this study were purchased from Sigma Chemical Company, and are not specifically listed in this section. Specialist materials and manufacturers are listed below.

Antibodies and Peptides

5E8 mouse monoclonal anti- α_2 integrin antibody (Chen et al., 1991) was a kind gift of Dr. R. Bankert, Roswell Park Cancer Institute, Buffalo, NY, USA; 6F1 mouse monoclonal anti- α_2 integrin antibody (Coller et al., 1989) was a kind gift of Dr. B.S. Coller, Mount Sinai Hospital, New York, NY, USA; LM609 mouse monoclonal anti- $\alpha_v\beta_3$ integrin antibody (Wayner et al., 1991) was purchased from Chemicon, USA; mouse IgG (Sigma) was used as an antibody control. RGD and RYD (single letter amino acid code) containing-peptides were purchased from Sigma.

Cell Lines and Tissue Culture

HT1080 K2P fibrosarcoma cells were a kind gift of C. Marshall, Institute of Cancer Research, London, UK. A375M melanoma cells were obtained from the European Cell and Culture Collection. Cells were maintained at 37°C, 5% CO₂, in Dulbecco's Modified Eagle's Medium (DMEM; GibcoBRL, UK) supplemented with 10% heat-inactivated foetal calf serum (FCS; Globepharm, USA) and the antibiotics penicillin and streptomycin (1000 iu/ml-1000 µg/ml, GibcoBRL, UK). Cells were passaged 24-48 hours prior to experimentation and harvested at 80% confluency using trypsin/EDTA solution (Sigma).

Electrophoresis, Silver Staining and Gelatin Zymography

Proteins were analysed by SDS-PAGE (Laemmli and Favre, 1973), using acrylamide solution, ammonium persulphate and potassium persulphate from BioRad Laboratories. SDS was purchased from Serva. Gels were formed and electrophoresed using BioRad mini-protean II equipment. (40% acrylamide, 0.01% Bis acrylamide solution)

Proteins were visualized using silver nitrate solution. Briefly, gels were fixed in 40% methanol, 10% acetic acid solution for 30 minutes. The fix was removed and the gels rinsed with distilled water. A small pinch of 61.5% $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, 38.5% $\text{K}_3\text{Fe}(\text{CN})_6$ mixture was added to ~20 mls water and added to the gel to reduce any proteins present. After 5 minutes this solution was removed and the gels washed with several changes of water until they became colourless. Silver nitrate solution (0.012M) was added to the gels and incubated for 20 minutes at room temperature with constant agitation. The silver nitrate solution was removed and the gels washed several times with water, prior to a single rinse in 2.5% Na_2CO_3 solution. Gels were developed with 100 μl of 38.5% paraformaldehyde solution in 100 mls of 2.5% Na_2CO_3 until bands appeared. Development was stopped using 5% acetic acid solution. Gels were dried in 10% glycerol, 5% acetic acid solution and preserved in porous acetate.

To analyse samples for gelatinase activity, heat-denatured (20 minutes at 60°C) type I calf skin collagen (0.5 $\mu\text{g}/\text{ml}$) was incorporated into 8% polyacrylamide SDS-PAGE gels (Heussen and Dowdle, 1980). Non-reduced samples were separated by electrophoresis and the gels washed twice with 2.5% Triton X-100 for 15 minutes per wash. Excess Triton was rinsed off with water and the gels incubated for 16 hours at room temperature (or 37°C) in 100mM Tris pH 7.9, 30 mM CaCl_2 , 0.04% Brij 35, 0.02% sodium azide. Gels were stained with Coomassie Brilliant Blue and then destained slightly to reveal bands of gelatin lysis. Zymograms were dried and preserved as for silver stained gels

Generation and Purification of Type I Collagen 3/4 and 1/4 Fragments

Rat skin type I collagen was isolated and purified as described by Cawston and Barrett (1979). Recombinant human collagenase-3 was expressed by stable transfection of NSO mouse myeloma cells and purified from serum-free conditioned cell culture medium (Knäuper et al., 1996a). Collagenase-3 was activated with p-aminophenylmercuric acetate (APMA) for 30 minutes at 37°C (Knäuper et al., 1996a).

Type I collagen (5 mg) in 50 mM Tris pH 7.4, 150 mM NaCl (Tris buffered saline, TBS) with 60 mM CaCl₂ was incubated with activated collagenase-3 (18 µg) for 16 hours at 25°C. Isolation of 3/4 and 1/4 type I collagen fragments from this cleavage mixture was performed essentially as described by Fitzsimmons et al. (1986) with minor modifications. The cleavage mixture was placed on ice at 4°C and solid ammonium sulphate added to 12% saturation to precipitate any uncleaved type I collagen. The mixture was stirred constantly for 1 hour on ice and centrifuged at 15,000 g for 1 hour at 4°C. The supernatant was removed, ammonium sulphate added to 18% saturation to precipitate the 3/4 fragment and stirred for 1 hour on ice. The 12% pellet was washed by resuspension in TBS containing 12% ammonium sulphate and repelleted by centrifugation at 15,000 g for 1 hour at 4°C. The pellet was solublized in 0.2 M acetic acid and dialysed for 16 hours into TBS. The precipitate produced by 18% ammonium sulphate precipitation was centrifuged as before, washed with TBS containing 18% ammonium sulphate, and solublized and dialysed as above. The ammonium sulphate concentration of the supernatant was raised to 25% to precipitate the 1/4 fragment and purified as above. The protein concentration of each solution was estimated by bicinchoninic acid (BCA) assay (Smith et al., 1985) (see below), following the manufacturers instructions (Pierce). A known concentration of each sample was analysed with uncleaved type I collagen by 5-15% polyacrylamide gradient gel electrophoresis and stained with silver reagent.

Adsorption of the fragments to plastic at room temperature stabilized the triple helical fragment conformation even when the fragment-coated plastic was later placed at 37°C for the duration of the assay (usually 30 minutes, as revealed by cell attachment assays performed at 37°C, see Chapter 5). It was therefore necessary to heat denature the collagen fragments for 16 hours at 35°C prior to assay at 37°C.

The presence of CL-3 on the collagen fragments was probed by ELISA using a sheep anti-human CL-3 polyclonal antibody produced in this laboratory (L29/6; Cowell, S., Knäuper, V., Stewart, M., d'Ortho, M.-P., Stanton, H., Hembry, R.M., López-Otín, C., Reynolds, J.J. and Murphy, G., paper in preparation). No detectable level of CL-3 was found in association with fragments adsorbed to ELISA plates (Maxisorp, Nunc), although a positive control of 10 ng/well CL-3 adsorbed alongside the fragments was readily detected by this method.

Immunolocalization

Cells were grown in DMEM supplemented with 10% FCS on glass coverslips coated with 20 µg/ml substrate (type I collagen, fragments of type I collagen or fibronectin; adsorbed to the glass for 16 hours at 4°C). Cells were then washed with serum-free DMEM and fixed with 4% paraformaldehyde in 0.5x PBS pH7.4, for 5 minutes. Coverslips were washed with fresh PBS and incubated with 0.1% Triton X-100 in PBS for 5 minutes to permeabilise cells. Cells were washed and incubated with 200 µl per/coverslip of 5 µg/ml primary, monoclonal antibody in PBS with 50 µg/ml blocking serum, (same species as the secondary antibody was raised in), for 30-60 minutes at room temperature. Cells were washed and incubated with appropriate fluorescent-labelled secondary antibodies diluted 1:200 (donkey-anti-mouse IgG Texas Red-conjugated antibody, Jackson ImmunoResearch Ltd (Texas Red is a registered trade mark of Jackson ImmunoResearch Ltd.); rabbit anti-rat IgG FITC-conjugated antibody, Dako) in

PBS for 30 minutes at room temperature. Cells were washed and mounted in Citifluor (City University, London) and examined under a fluorescence microscope (Zeiss Photomicroscope III; Carl Zeiss, New York). Photographs were taken using Kodak Panther 1600 ASA film.

MMP Activity Assays

Gelatinase activity was measured using the method of Murphy et al. (1981b). Briefly, ^{14}C -acetylated rat skin type I collagen (1 mg/ml) was denatured at 60°C for 20 minutes and placed in tubes, (100 μl /tube), with samples and 100 mM Tris pH 7.6, 10 mM CaCl_2 , 0.02% NaN_3 , 0.04% Brij 35 (TCAB) to a total volume of 250 μl for 16-18 hours at 37°C. Undegraded gelatin was precipitated with 30% trichloroacetic acid (50 μl /tube) for 30 minutes on ice. The precipitate was pelleted by centrifugation for 15 minutes at 12,000 g and 4°C, and the radioactivity of 200 μl /sample determined by scintillation counting. Incubation of 100 μl of gelatin with either 16 μg bacterial collagenase or 10 μg trypsin, controlled for total lysis of the gelatin and gelatin integrity respectively. The gelatinase activity of the samples was converted into the linear range of the assay (20-75% of total lysis) and expressed as units/ml of enzyme activity.

Stromelysin activity was detected using 1 mg/ml ^{14}C -acetylated casein (100 μl /tube) by the method of Murphy et al. (1981b), essentially as described for gelatinase assays. However, undegraded casein was precipitated using 50 μl /tube 3% trichloroacetic acid on ice for 30 minutes prior to pelleting by centrifugation. Bacterial collagenase controls were omitted from these assays and total lysis of the casein determined using 10 μg /tube trypsin, as described above. Results were analysed and expressed as described for gelatinase assays.

Collagenase activity was detected by the method of Cawston and Barrett (1979), which is similar to the gelatinase assay detailed above. Briefly, 100 μl of 1 mg/ml ^{14}C -

acetylated rat skin type I collagen was incubated with samples and TCAB in a total volume of 300 μ l/tube. The tubes were incubated for 16-18 hours at 35°C and uncleaved collagen pelleted by centrifugation for 15 minutes at 12,000g at 4°C. The radioactivity of 200 μ l of each sample was determined by scintillation counting. Control lysis with bacterial collagenase and trypsin were performed as described for gelatinase assays. Results were analysed and expressed as described for gelatinase assays.

All samples were assayed in triplicate for all MMP assays, and one unit of enzyme degrades 1 μ g of substrate in 1 minute at 37°C.

Protein Estimation

The concentration of protein samples was estimated using the bicinchoninic acid method (Smith et al., 1985) according to the manufacturers (Sigma) instructions. Briefly, bicinchoninic acid (50 parts) was mixed with copper (II) sulphate (1 part) to produce the protein determination reagent. 2 mls of protein reagent was mixed with known volumes of protein standard diluted in water (to form a calibration curve), and with the unknown protein sample. The total volume of each tube did not exceed 2.1 mls. Samples were incubated at 37°C for 30 minutes then allowed to cool to room temperature. Once cool, the absorbance of the samples at 562 nm was determined using a spectrophotometer. The absorbance of known quantities of protein standard were plotted to give a standard curve, from which the concentration of the unknown samples was determined. In all cases, 1 mg/ml BSA was used as the protein standard.

Recombinant Human Matrix Metallproteinases and Inhibitors

Procollagenase-3 was a kind gift from Dr. Vera Knäuper (University of East Anglia, Norwich, UK). Progelatinase A (Crabbe et al., 1993), progelatinase B (O'Connell et al., 1994), procollagenase-1 and stromelysin-1 (Murphy et al., 1992b) were kind gifts

from Prof. Gillian Murphy (University of East Anglia, Norwich, UK). TIMP-2 (Willenbrock et al., 1993) was a gift from Dr. Mike Hutton (University of East Anglia, Norwich, UK). Full length MT-1 MMP and MT-2 MMP were gifts from Dr. Horst Will (In Vitek GmbH, Berlin-Buch, Germany).

Chapter 3

Interaction of Isolated $\alpha_2\beta_1$ Integrin With Type I Collagen and Collagen Fragments

Introduction

To study effect of collagenase-degradation on cell-surface receptor interactions with monomeric type I collagen, solubilised collagen was incubated overnight with active collagenase-3 to produce the classical 3/4 and 1/4 length fragments. These fragments were purified from the residual, uncleaved collagen, thereby ensuring that all receptor-collagen interactions could be attributed to the collagen fragments. In order to facilitate the detailed study of $\alpha_2\beta_1$ integrin mediated interactions with type I collagen and the collagenase-3 derived fragments of collagen, $\alpha_2\beta_1$ integrin was isolated from outdated human platelet preparations. These cells were chosen because $\alpha_2\beta_1$ integrin mediates platelet adhesion to monomeric type I collagen (Staatz et al., 1989; Santoro and Zutter, 1995), and because suitable quantities of outdated preparations were readily available.

Most reports of cell interactions with collagen fragments have concentrated on location of $\alpha_2\beta_1$ integrin-binding sites within the type I collagen molecule. This has been principally been studied using peptide fragments generated by cyanogen bromide (CNBr) cleavage of type I collagen (Fitzsimmons et al., 1986; Staatz et al., 1990; Gulberg et al., 1992; Morton et al., 1994), although Fitzsimmons et al. (1986) also used collagenase-1 generated collagen fragments.

Whilst the small peptides generated by CNBr cleavage of collagen are useful in attempting to pinpoint regions of the collagen molecule involved in receptor binding, they do not represent a physiological substrate for cell-surface receptors such as $\alpha_2\beta_1$

integrin. Collagenases, such as collagenase-3 (CL-3), are synthesised and secreted by many cell types *in vivo*, and are therefore more useful tools than CNBr for analysing the effects of degradation of the collagen molecule on cell-collagen interactions. As discussed in chapter 1, ECM remodeling has been shown to have a significant effect on cell behaviour. However, few studies on the effects of physiological proteolysis of fibrillar collagens on cell-matrix interactions have been published to date. The data presented in this chapter analyses the interactions of isolated collagen fragments and the $\alpha_2\beta_1$ integrin receptor, and compares these with $\alpha_2\beta_1$ integrin interactions with native type I collagen.

Methods

Reagents

Mouse monoclonal antibodies to human α_2 integrin (6F1 and 5E8) and a rat monoclonal to human β_1 integrin (mAb 13) were obtained as described in Chapter 2. Rat skin type I collagen and gelatin was prepared as described in Chapter 2. Collagenase-3 generated fragments of rat skin type I collagen were purified as described in Chapter 2.

Purification of $\alpha_2\beta_1$ Integrin From Outdated Human Platelets

This was carried out essentially as described by Vanderberg et al. (1991) with some modifications. Briefly, outdated human platelet concentrate apheresis packs of single ABO blood group were combined and centrifuged at 1,500 g for 20 minutes at 4°C. The supernatant was discarded and the pellets resuspended in 13 mM sodium citrate, 120 mM NaCl, 30 mM glucose pH 7.0 at 4°C. The suspension was centrifuged at 1,500 g for 20 minutes at 4°C and the washing procedure repeated as above. The resulting pellets were resuspended in 10 mM Tris pH 7.4, 5 mM sodium citrate, 100

μ M pepstatin A, 5 μ M trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), 2 μ g/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF) and sonicated at 30% output for 3x10 seconds on ice. The sonicates were centrifuged at 100,000 g for 30 minutes at 4°C and the supernatant discarded. The platelet membrane preparations were then either stored at -80°C until needed or lysed directly by resuspending in a small volume of 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1 mM MnCl₂, 100 mM n-octyl β -D-glucopyranoside (OG), 100 μ M pepstatin A, 5 μ M E-64, 2 μ g/ml leupeptin, 1 mM ABBSF (extraction buffer) for 30 minutes on ice. The lysate was centrifuged at 100,000 g for 30 minutes at 4°C and the supernatant collected and loaded directly onto a prepared collagen-Sepharose column.

Type I collagen (2.25 mg/ml) was coupled to 6-aminohexanoic acid n-hydroxysuccinimide ester linked, cyanogen bromide activated Sepharose 4B (Sigma) according to the manufacturers instructions. This was washed alternatively with three washes of: (a) 0.1M sodium acetate pH 4.0, 0.5M NaCl and (b) 0.1 M Tris pH 8.0, 0.5 M NaCl. The gel was washed with 50 mM Tris pH 7.4, 150 mM NaCl (TBS) and packed into a column. The column was equilibrated with two column volumes of TBS, 2 mM MgCl₂, 1 mM MnCl₂, 25 mM OG (equilibration buffer) followed by two column volumes of extraction buffer at a flow rate of 30 milliliters/hour. The platelet membrane supernatant was loaded onto the column at 10 milliliters/hour and the runoff reapplied to the column. One column volume of extraction buffer was then run through the column followed by at least two column volumes of equilibration buffer. Bound $\alpha_2\beta_1$ integrin was eluted off the column with TBS, 25 mM OG, 20 mM EDTA at a flow rate between 3-10 milliliters/hour. Protein eluted in the presence of EDTA were monitored by spectrophotometry at 280nm. Fractions containing protein were combined and dialysed into TBS, 2 mM MgCl₂, 1 mM MnCl₂, 0.02% sodium azide, and the protein concentration determined by BCA assay (Chapter 2). The purified integrin was sometimes biotinylated using a protein biotinylation kit (Amersham Life Sciences)

according to the manufacturer's instructions. All integrin preparations were aliquoted and stored at -80°C .

Characterization of the Purified Platelet $\alpha_2\beta_1$ Integrin

The purity of the affinity-purified collagen receptor was analysed by SDS-PAGE. Proteins were visualized by staining with silver nitrate (described in Chapter 2). Immunoprecipitation of the integrin with P1E6 anti- α_2 integrin antibody (Carter et al., 1990) was carried out essentially as described by Adams and Watt (1988). Briefly, biotinylated $\alpha_2\beta_1$ integrin was incubated with the antibody, then the integrin-antibody mixture incubated with protein A coupled to Sepharose matrix. Unbound protein was washed off the matrix and bound protein released by boiling with x2 reducing sample buffer. The sample was subjected to 6% SDS-PAGE and transferred onto Hybond ECL (Amersham Life Sciences) nitrocellulose membrane. The membrane was blocked with 5% non-fat milk powder in PBS with 0.1% Tween X-100 (PBS-Tween), washed in milk-free PBS-tween solution and incubated for 1 hour with streptavidin-horseradish peroxidase (Amersham Life Sciences: 1:1500 dilution) in PBS-Tween. Unbound streptavidin-peroxidase was washed off with PBS-Tween, and bound proteins visualized onto ECL Hyperfilm (Amersham Life Sciences) using SuperSignal Chemiluminescent Substrate and SuperSignal Stable Peroxidase solutions (Amersham Life Sciences).

Western blotting of the purified $\alpha_2\beta_1$ integrin was performed essentially as described by Towbin et al. (1979). Briefly, $\alpha_2\beta_1$ integrin was subjected to 6% SDS-PAGE and transferred onto Hybond ECL (Amersham Life Sciences) nitrocellulose membrane. Unbound membrane was blocked by incubation for 1 hour at 37°C with 5% non-fat milk powder in Western wash buffer. The blocked membrane was incubated for 16 hours at 4°C with mAb 13 anti- α_2 integrin antibody (Akiyama et al., 1989). Unbound primary antibody was washed off with wash buffer and the membrane blocked for a

second time at 37°C with 5% milk powder solution. The membrane was then incubated with horseradish-peroxidase conjugated, rabbit-anti-rat IgG secondary antibody in wash buffer for 1 hour at room temperature. Unbound secondary antibody was washed off with wash buffer and the membrane-bound proteins visualized with ECL solutions, as described for the immunoprecipitation protocol above.

Solid-phase Ligand Binding Assays

Experiments in which the $\alpha_2\beta_1$ integrin receptor was adsorbed to 96 well ELISA plates (Maxisorb, Nunc), and biotinylated ECM ligands were added are termed "receptor-immobilized" ligand binding assays and were performed as described by Kern et al. (1993). Briefly, 100 μ l/well of $\alpha_2\beta_1$ integrin (1.75 μ g/ml) in TBS, 1 mM MgCl_2 , 1 mM MnCl_2 , 0.02% sodium azide (buffer A) was adsorbed to ELISA plates for 16 hours at 4°C and unbound plastic was blocked by incubation with buffer A containing 1% BSA (buffer B, 100 μ l/well) for 1 hour at room temperature. Where antibodies were used, these were added to washed wells in buffer B at a total volume of 100 μ l/well and incubated for 1 hour at room temperature. Wells were washed 3 times with buffer B and 100 μ l/well of biotinylated type I collagen and type I collagen fragments were incubated with the immobilized integrin for 2 hours at room temperature, with or without 10 mM EDTA. After washing the wells three times with buffer B, bound proteins were detected by incubation with streptavidin-horseradish peroxidase (Amersham Life Sciences; 1:1500 dilution) in buffer B (100 μ l/well) for 1 hour at room temperature. Unbound streptavidin was washed off the wells three times with buffer A and bound peroxidase activity was measured by incubation with 3,3',5,5'-tetramethylbenzidine-peroxidase (TMB) substrate mixed 1:1 with H_2O_2 (100 μ l/well). The colour reaction was stopped using 50 μ l/well 2.5 M H_2SO_4 and the absorption of the solution in the wells was measured at 450 nm using an ELISA reader.

Due to the difference in size of the whole collagen molecule and the 3/4 and 1/4 collagen fragments, it was necessary to determine the degree of biotinylation of each of these ligands. Serial dilutions of biotinylated proteins were dotted onto Hybond ECL nitrocellulose membrane (Amersham Life Sciences) and incubated with streptavidin-horseradish peroxidase (Amersham Life sciences; 1:1500) in TBS for 30 minutes at room temperature. Peroxidase activity was detected using SuperSignal Chemiluminescent Substrate and Stable Peroxidase (Amersham Life Sciences) ECL reagents and the blot exposed to Hyperfilm (Amersham Life Sciences) ECL film for 15-30 seconds. The film was developed and scanned into a Leica Q500+ image analyser (in collaboration with Dr. Richard Farndale, Department of Biochemistry, University of Cambridge, Cambridge, UK) and the density of each dot of protein measured by the image analyser. Values were corrected against the background and mean values for each protein compared. OD values obtained during "receptor-immobilized" ligand binding assays were then corrected for the degree of biotinylation of each ligand.

Experiments in which type I collagen and type I collagen fragments were adsorbed to ELISA plates and then biotinylated $\alpha_2\beta_1$ integrin added are termed "ligand binding assays". For experiments carried out at room temperature, type I collagen and the fragments in TBS (100 μ l/well) were allowed to bind to the ELISA plates for 1 hour at room temperature and uncoated plastic blocked with 50 mg/ml BSA in TBS (100 μ l/well) under the same conditions. Wells were washed three times with TBS, 1 mM $MgCl_2$, 1 mM $MnCl_2$ (buffer C) and incubated with biotinylated $\alpha_2\beta_1$ integrin in 1 mg/ml BSA in buffer C (buffer D; 100 μ l/well) for 2 hours at room temperature. Where antibodies or EDTA were used, these were added to the wells at the same time as the integrin. Unbound integrin was removed and wells washed three times with buffer C. Wells were incubated with streptavidin-horseradish peroxidase (1:1500 dilution) in buffer D (100 μ l/well) for 15-20 minutes at room temperature and bound protein visualised as described for the "receptor-immobilized" ligand binding assays.

For studies of $\alpha_2\beta_1$ integrin interactions with the denatured fragments, ligand binding assays at 37°C were performed essentially as described above. However, the biotinylated collagen fragments were incubated at 35°C for 16 hours prior to coating to warmed ELISA plates for 1 hour at 37°C. Blocking and wash stages were performed at 37°C using the buffers described above, warmed to 37°C. Incubation with biotinylated ligands was carried out at 37°C for 30 minutes. Washed wells were incubated with streptavidin-horseradish peroxidase for 15 minutes at 37°C and bound proteins were visualised as described for the "receptor-immobilized" ligand binding assays.

Results

The purity of the type I collagen fragments generated by CL-3 cleavage of native type I collagen was determined by 6% SDS-PAGE followed by silver staining. Each fragment was found to be essentially free of contamination with uncleaved collagen or the other fragment (fig.3.1)

The purity of the human platelet $\alpha_2\beta_1$ integrin preparation was determined by 6% polyacrylamide gel electrophoresis and silver staining. Two bands were resolved on the gel, of approximate molecular mass of 150 kDa and 120 kDa (fig.3.2), which correspond to the published molecular mass of the α_2 and β_1 integrin subunits respectively analysed under reducing conditions (Takada and Hemler, 1989; Vanderberg et al., 1991). The bands were further characterized by immunoprecipitation with P1E6 anti- α_2 integrin antibody (Carter et al., 1990). Two bands were visualized by immunoprecipitation, (of approximate mass of 150 kDa and 125 kDa), as the integrin α and β subunits form a tight complex which precipitates both subunits when probed with an antibody to a single subunit (fig.3.3). Western blotting using mAb 13 anti- β_1 integrin antibody (Akiyama et al., 1989) located a single band of approximate molecular mass of 125 kDa (fig.3.4), corresponding to the described molecular mass of the β_1 subunit.

Biotinylated type I collagen bound to immobilized $\alpha_2\beta_1$ integrin in a dose-dependent manner (fig.3.5). Collagen binding to $\alpha_2\beta_1$ integrin was inhibited by incubation of the immobilized integrin with 6F1 anti- α_2 integrin and mAb 13 anti- β_1 integrin antibodies prior to addition of the collagen, whereas a control IgG had no effect (fig.3.6). This result demonstrated the specificity of the biotinylated collagen binding to the $\alpha_2\beta_1$ integrin. Collagen binding to the immobilized integrin could also be completely inhibited by 10 mM EDTA (fig.3.6), confirming the that this was a cation-dependent interaction. Biotinylated 3/4 and 1/4 type I collagen fragments were found to bind to immobilized $\alpha_2\beta_1$ integrin approximately 67% and 15% as well as full length collagen respectively when coated at the same molar concentration (fig.3.7). The correction for the degree of biotinylation with respect to each collagenous ligand was determined from densitometric analysis of a serial dilution of each ligand (fig.3.8).

Biotinylated $\alpha_2\beta_1$ integrin bound to immobilized type I collagen in a dose-dependent manner (fig.3.9). $\alpha_2\beta_1$ integrin binding to immobilized collagen and fragments was compared at room temperature and it was found that $\alpha_2\beta_1$ integrin bound equally well to native collagen and the native 3/4 fragment of type I collagen. Less integrin binding to the 1/4 fragment was observed at lower ligand concentrations, however, binding increased to approximately 60% of that observed to native collagen at the highest ligand concentration tested (fig.3.10). The $\alpha_2\beta_1$ integrin binding to the native type I collagen and the fragments was completely inhibitable by 10 mM EDTA, indicating that the binding was most likely to be integrin-mediated (fig.3.10).

The $\alpha_2\beta_1$ integrin binding to native collagen and the native fragments which was observed at room temperature could be significantly reduced by addition of 6F1 and 5E8 anti α_2 integrin antibodies, but a control IgG had no effect on integrin-ligand binding (fig.3.11). This confirmed the specificity of the biotinylated $\alpha_2\beta_1$ integrin interaction with the collagen fragments.

$\alpha_2\beta_1$ integrin binding to native collagen and denatured collagen fragments at 37°C was also tested. The results showed the necessity for retention of the collagen fragment triple-helical structure to maintain $\alpha_2\beta_1$ integrin interactions, since heat-denaturation of the fragments completely inhibited $\alpha_2\beta_1$ integrin binding (fig.3.12). Heat-denaturation of type I collagen also inhibited $\alpha_2\beta_1$ integrin binding at 37°C compared to integrin binding to native collagen, although at the highest gelatin concentration a low level of $\alpha_2\beta_1$ integrin was observed (fig.3.13).

Discussion

The data presented in this chapter strongly indicate that $\alpha_2\beta_1$ integrin binding to type I collagen fragments, generated by *in vitro* degradation by collagenase-3, is totally dependent on retention of the collagen triple-helix. The implications of this result for $\alpha_2\beta_1$ integrin interactions with collagen fragments generated *in vivo* are profound, as these fragments might be expected to denature at 37°C as soon as they were formed, leading to abolition of $\alpha_2\beta_1$ integrin binding.

Two types of ligand-binding assays were employed to test isolated $\alpha_2\beta_1$ integrin interactions with type I collagen and the 3/4 and 1/4 fragments. Pfaff et al. (1994) found that stronger integrin-ligand binding was observed when the isolated integrins were immobilized on ELISA plates and the ligands added in solution than when the ECM ligands were coated onto ELISA plates and the integrins added in solution. This is possibly due to integrin ~~conformation being optimised~~ ^{the conformation} when adsorbed onto plastic compared to α in solution, therefore allowing the integrins to function more readily as ECM protein receptors. A study by Kern et al. (1993), which used platelet $\alpha_2\beta_1$ integrin purified in a similar manner as described below, analyzed the coating efficiency of this integrin using iodinated integrin and found that 50-60% of the integrin coated in solution was immobilized on the ELISA plates by adsorption.

Due to complications in analysing the data generated from this type of "receptor-immobilized" assay, ligand binding assays were also performed using immobilized type I collagen and fragments and biotinylated $\alpha_2\beta_1$ integrin. This allowed direct comparison of integrin binding to each substrate without correction for degree of biotinylation of the substrates, and also permitted less variation in results between integrin and fragment preparations.

The majority of biotinylated $\alpha_2\beta_1$ integrin binding to collagen fragments was supported by the native 3/4 fragment of type I collagen. This was essentially the same level of binding as was observed to the whole collagen molecule. Since $\alpha_2\beta_1$ integrin only appeared to interact with the 1/4 fragment of type I collagen at high coating concentration at room temperature, it is possible that this fragment does not contain many $\alpha_2\beta_1$ integrin binding sites. This is in agreement with data with cyanogen bromide derived fragments of type I collagen (Morton et al., 1994). The 1/4 fragment generated by collagenase cleavage of type I collagen is equivalent to a small section of $\alpha 1(I)C\text{NBr } 7$ and all of $C\text{NBr } 6b$ (Gross et al., 1980; see Chapter 1, fig.1.3). No human platelet adhesion to $C\text{NBr } 6b$ via $\alpha_2\beta_1$ integrin was observed, and although $C\text{NBr } 7$ was found to support platelet adhesion, the collagenase-generated 1/4 fragment may not contain enough of the $C\text{NBr } 7$ region to actively support α_2 integrin interactions. However, it is possible that the 1/4 fragment was partially denatured during the assays at room temperature, thus reducing any integrin-mediated interactions. This is unlikely as the published melting temperature of this fragment (29°C; Stark and Kühn, 1968a) is several degrees higher than the assay temperature. Binding to the triple helical fragments was significantly reduced by anti- α_2 integrin antibodies at the concentrations used, and completely abrogated by EDTA, confirming that binding to collagen and the fragments was specifically mediated by $\alpha_2\beta_1$ integrin.

Studies by Stark and Kühn (1968a) showed that the 1/4 and 3/4 fragments of type I collagen lose their triple helical structure below physiological temperature. Thus, collagenase cleavage of type I collagen may be an instance of *in vivo* generation of gelatin. The results presented above show that $\alpha_2\beta_1$ integrin was able to bind to the triple helical 3/4 and 1/4 fragments of collagen, but not to the heat-denatured fragments, which were essentially gelatin. The results extend the data published by Morton et al. (1994), where the authors found that $\alpha_2\beta_1$ integrin-mediated platelet adherence to CNBr fragments was ablated at 37°C. These data underscore the need for native, triple helical collagen conformation as a prerequisite for $\alpha_2\beta_1$ integrin mediated binding, and strongly suggest that $\alpha_2\beta_1$ integrin does not mediate cell interactions with cleaved type I collagen *in vivo*.

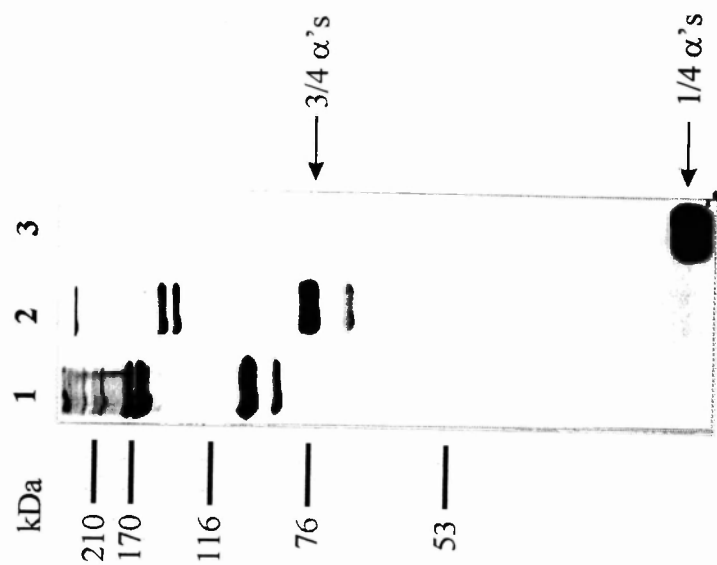


Fig. 3.1: Silver stain of native type I collagen (lane 1) and the purified 3/4 fragment (lane 2) and 1/4 fragment (lane 3) generated by collagenase-3 cleavage of type I collagen. Collagenase cleaves the collagen α chains at approximately 3/4 of the length of the collagen molecule from the N terminus.

Fig.3.2

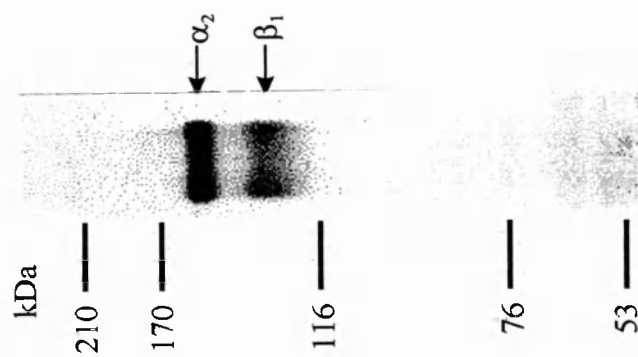
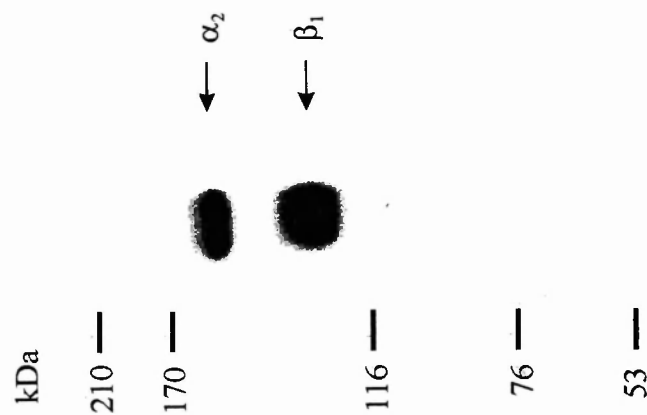


Fig.3.3



Silver stain (fig.3.2) and immunoprecipitation (fig.3.3) of $\alpha_2\beta_1$ integrin purified from human platelets. Protein bands which correspond to the published size of the $\alpha_2\beta_1$ integrin are marked on the gel and blot.

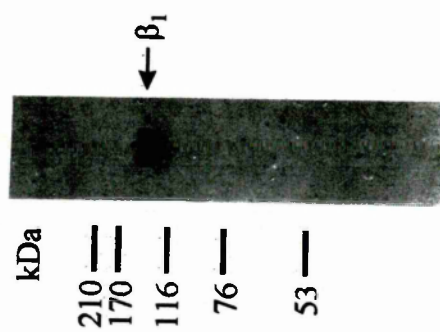


Fig.3.4: Western blot of $\alpha_2\beta_1$ integrin isolated from human platelets. The position of the single protein which bound to mAb 13 anti- β_1 integrin antibody corresponds to the published size of the β_1 integrin subunit.

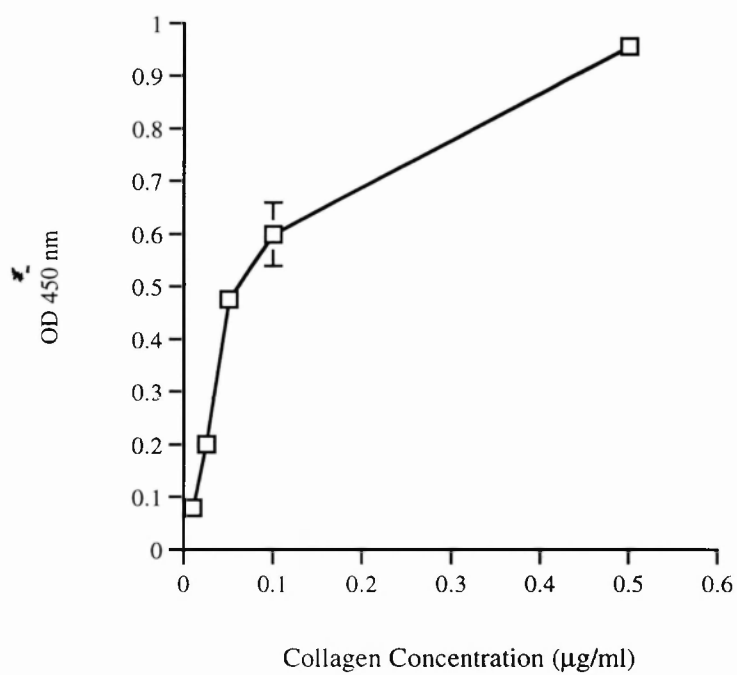


Fig.3.5 : Biotinylated type I collagen binding to immobilized $\alpha_2\beta_1$ integrin increased with collagen concentration. Values shown are the mean of triplicates, +/- standard deviation. Where no error bars are visible, the bars are smaller than the point marker.

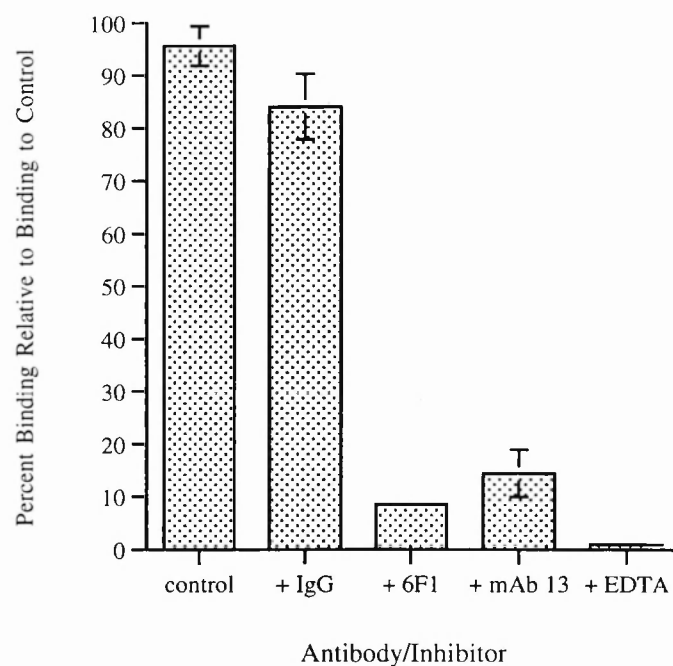


Fig.3.6 : Biotinylated type I collagen (0.1 $\mu\text{g/ml}$) binding to immobilized $\alpha_2\beta_1$ integrin (1.75 $\mu\text{g/ml}$) was inhibited by preincubation of bound integrin with 6F1 anti- α_2 integrin antibody (5.0 $\mu\text{g/ml}$), mAb13 anti- β_1 integrin antibody (10 $\mu\text{g/ml}$). A control IgG (10 $\mu\text{g/ml}$) had no effect on collagen binding. EDTA (10 mM) completely inhibited type I collagen binding. Data marked "control" refers to biotinylated collagen binding in the absence of inhibitors. Values are the mean of triplicates, \pm standard deviation. Where no error bars are visible, bars are too small to be seen on this scale.

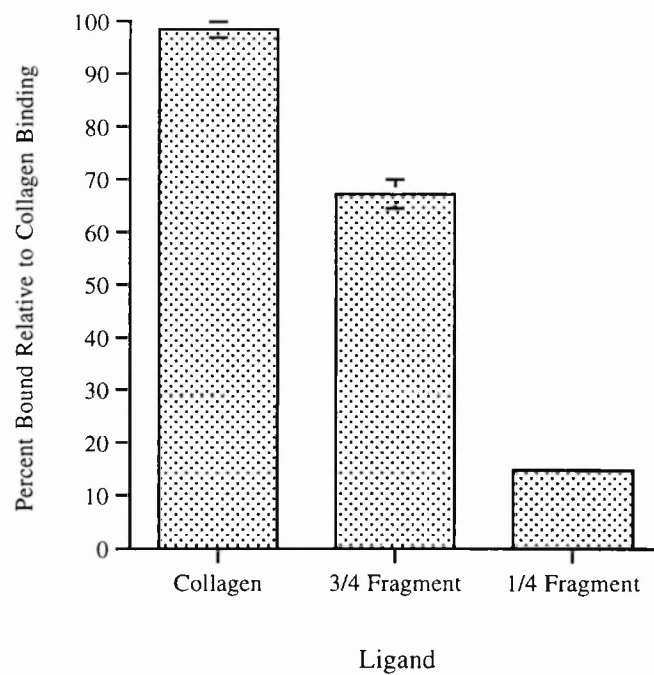


Fig.3.7 : Biotinylated, native type I collagen and collagen fragments (0.1 $\mu\text{g/ml}$) bound to immobilized $\alpha_2\beta_1$ integrin (1.75 $\mu\text{g/ml}$) at room temperature. The 3/4 fragment bound approximately 67% as well as the whole collagen molecule, however the 1/4 fragment bound only 15% as well as collagen. Values shown are corrected for the degree of biotinylation of each ligand and are the mean of triplicates, \pm standard deviation. Where no error bar is visible, the bar is too small to be visible on this scale.

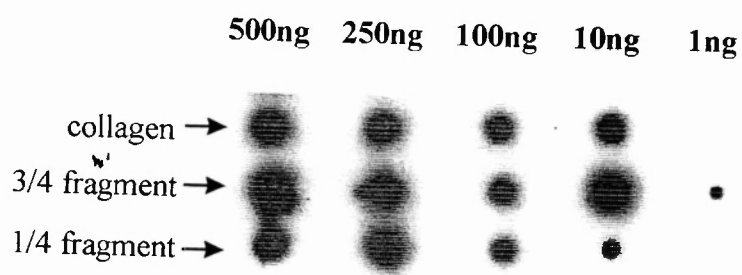


Fig.3.8: Dot blot of biotinylated type I collagen and collagen fragments. Serial dilution of the biotinylated proteins revealed that for this batch of proteins that more biotin had bound to the 3/4 fragment than the 1/4 fragment or full length collagen. The blot was analysed by densometric scanning and values corrected for the degree of biotinylation.

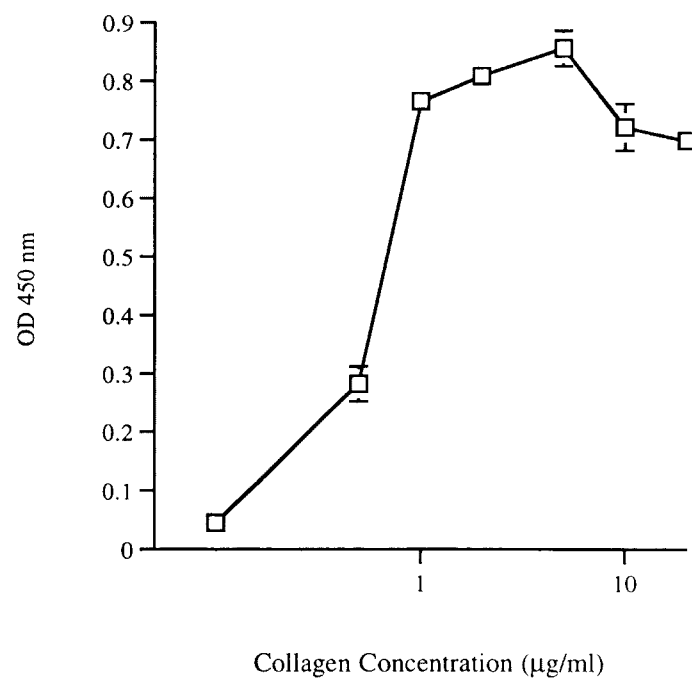


Fig.3.9 : Biotinylated $\alpha_2\beta_1$ integrin binding to immobilized type I collagen increased with increasing integrin concentration. Values shown are the mean of triplicates, +/- standard deviation. Where no error bars are visible, the bars are smaller than the point markers.

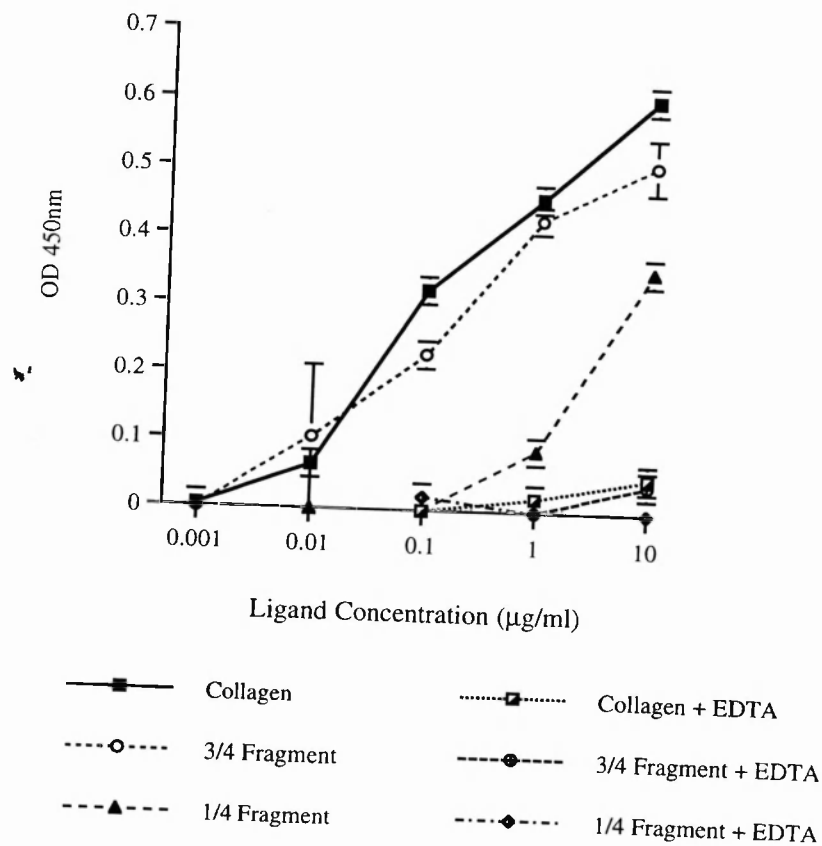


Fig.3.10 : Biotinylated $\alpha_2\beta_1$ integrin bound equally well to native collagen and the 3/4 fragment of type I collagen at room temperature, rising with increasing ligand concentration. Integrin binding to the native 1/4 fragment was only observed at high ligand coating concentration. Integrin binding to all the ligands was inhibited by addition of 10 mM EDTA. Values shown are the mean of triplicates, +/- standard deviation.

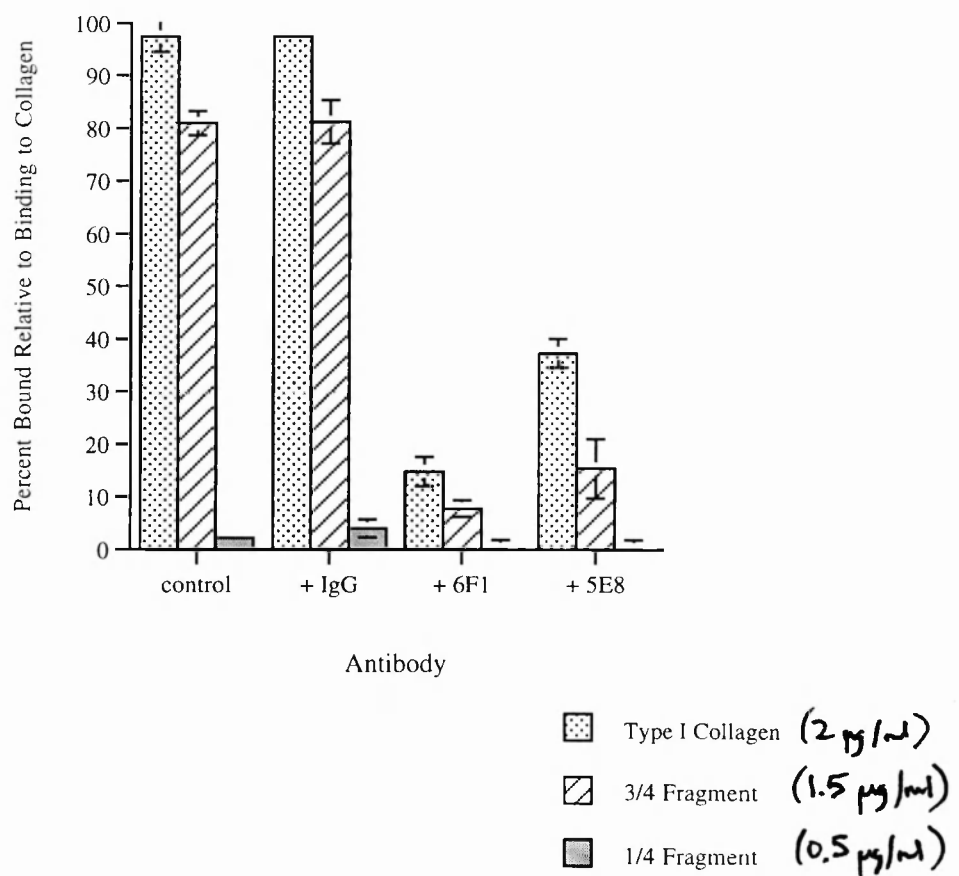


Fig.3.11 : Biotinylated $\alpha_2\beta_1$ integrin binding to type I collagen and the fragments was inhibited by incubation with 6F1 and 5E8 anti- α_2 integrin antibodies but not by mouse IgG (5.0 $\mu\text{g/ml}$). Collagen and fragments were coated on an equimolar basis for direct comparison. Values marked "control" refer to integrin binding in the absence of antibodies. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are too small to be visible on this scale.

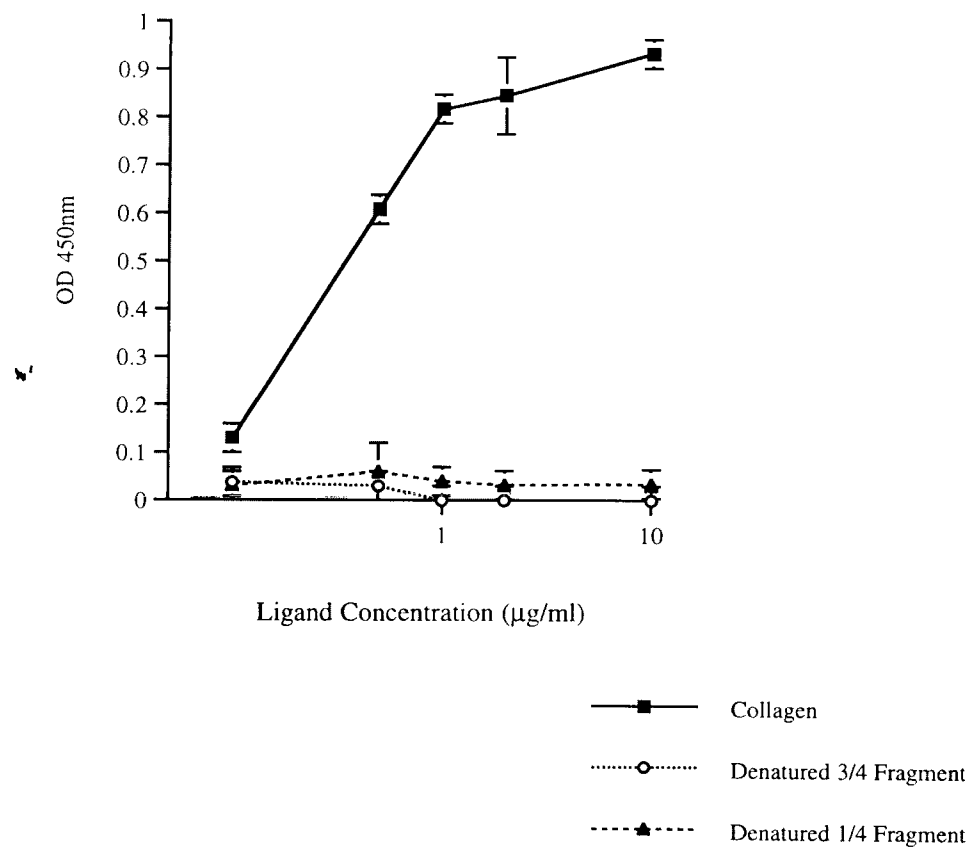


Fig.3.12 : Heat-denaturation of type I collagen 3/4 and 1/4 fragments completely ablated biotinylated $\alpha_2\beta_1$ integrin binding at 37 °C. Integrin binding to native collagen was still possible at this temperature. Values shown are the mean of triplicates, +/- standard deviation.

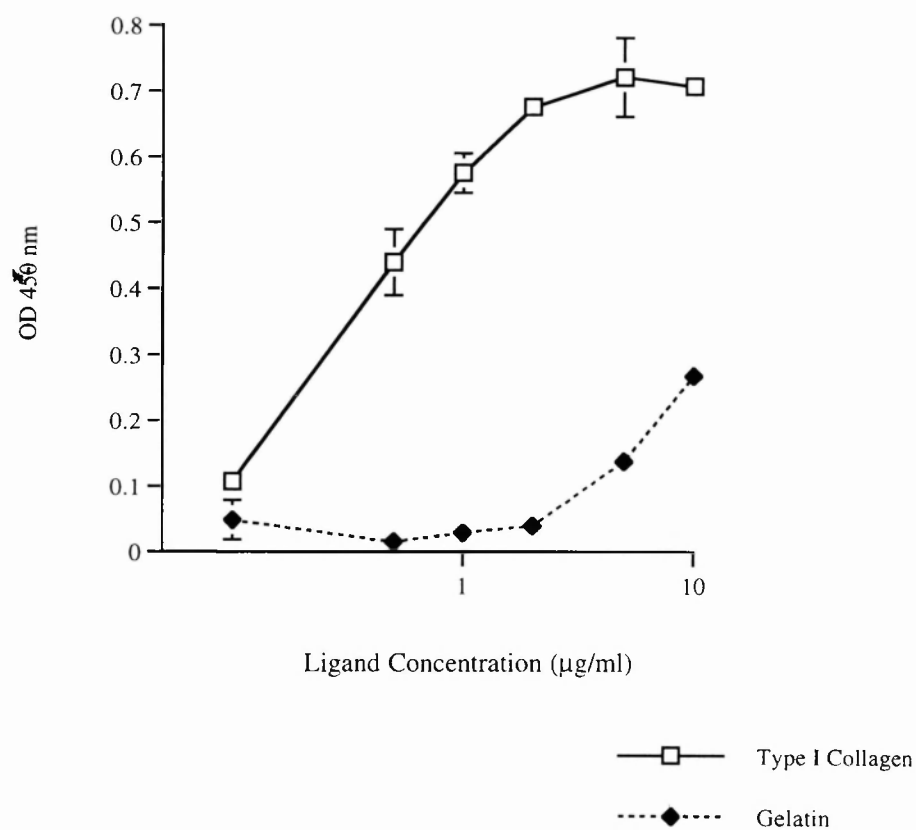


Fig.13 : Binding of biotinylated $\alpha_2\beta_1$ integrin to native type I collagen at 37°C increased with increasing collagen concentration, however, integrin binding to heat-denatured collagen (gelatin) was only observed at high ligand coating concentrations. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not visible, bars are smaller than the point markers.

Chapter 4

α_2 Integrin A-domain Binding to Type I Collagen and Collagen Fragments

Introduction

The α_2 integrin is one of several alpha integrin subunits (α_1 , α_2 , α_L , α_M , α_X and α_E) which have been found to contain a ~200 amino acid inserted domain (I-domain) between repeats II and III of the seven-fold repeated N-terminal domain structure (Hughes, 1992; Shaw et al., 1994). This region is similar in sequence to the collagen binding motif found in the A-domain of von Willebrand factor (Pareti et al., 1987) and ^{the} A-domain-like sequences found in type VI collagen, complement factor B and cartilage matrix protein (Colombatti et al., 1993). The α_2 integrin A-domain has been shown to the collagen-binding region of the $\alpha_2\beta_1$ integrin (Kamata and Takada, 1994; Tuckwell et al., 1995), and it was therefore of interest to study the effects of collagenase-cleavage of collagen on A-domain binding. It is unclear whether divalent cations such as Mg^{2+} or Mn^{2+} are essential for all A-domain function, however, the α_2 integrin A-domain requires divalent cations to interact with type I collagen (Tuckwell and Humphries, 1996b). The purpose of this investigation was to confirm and extend the data generated during the study of whole $\alpha_2\beta_1$ integrin interactions with type I collagen and type I collagen fragments in their native and denatured conformation.

Methods

Antibodies and Reagents

Mouse monoclonal antibodies to human α_2 integrin (6F1 and 5E8) were obtained as described in Chapter 2. Recombinant human α_2 integrin A-domain was a kind gift from Dr. Danny Tuckwell, School of Biological Sciences, University of Manchester, Stopford Building, Oxford Road, Manchester, UK. Rat skin type I collagen was prepared as described in Chapter 2. Collagenase-3 generated fragments of rat skin type I collagen were purified as described in Chapter 2.

Generation and Labelling of Recombinant α_2 Integrin A-domain

Recombinant α_2 integrin A-domain was produced by D. Tuckwell at Manchester University, as described by Tuckwell et al. (1995a). Briefly, human α_2 integrin A-domain cDNA was generated by reverse transcription/PCR from HT1080 cell RNA, cloned into the pGEX-2T vector (Smith and Johnson, 1988) and transfected into *E. coli* DH5 α F' bacteria. Induction of bacteria that carried the plasmid with 0.1 mM isopropyl- β -D-thiogalactoside led to production of α_2 integrin A-domain-glutathione-S-transferase (GST) fusion protein, which was purified from bacterial lysates on a glutathione-affinity column. The fusion protein was dialysed into TBS to remove the glutathione and cleaved with thrombin (Sigma; 1:100 (w/w) enzyme:fusion protein). The cleavage mixture was reduced with dithiothreitol (DTT) and passed a second time down a glutathione agarose column to remove GST. The purified α_2 integrin A-domain was dialysed into TBS and stored at -80°C .

Recombinant α_2 integrin A-domain was biotinylated using a protein biotinylation kit (Amersham Life Sciences). Briefly, A-domain was dialysed in to PBS and incubated with biotinylation reagent for 1 hour at room temperature, following the manufacturer's instructions. Unbound biotin was removed by extensive dialysis into TBS, 1 mM MgCl_2 (at least 3 changes of buffer over 48 hours) and the protein concentration of the solution determined by BCA assay as described in Chapter 2.

Solid-phase Ligand Binding Assays

These were performed essentially as described by Tuckwell et al. (1995a). For experiments carried out at room temperature, type I collagen, gelatin or type I collagen 3/4 and 1/4 fragments (100 µl/well) were coated to 96 well ELISA plates (Maxisorb, Nunc) by adsorption for 1 hour at room temperature. Unbound substrate was flicked off and the wells blocked with 100 µl/well of 50 mg/ml BSA in TBS for 1 hour at room temperature. Wells were washed three times with TBS, 1 mM MgCl₂ and biotinylated A-domain was added to wells in the presence or absence of antibodies or 10 mM EDTA (total volume: 100 µl/well) for 3 hours at room temperature. Unbound A-domain was flicked off and wells washed three times as described above. Wells were incubated with 100 µl/well streptavidin horseradish peroxidase (1:1500; Amersham Life Sciences, part of the biotinylation kit) for 20 minutes at room temperature, washed as before and bound streptavidin visualised using TMB reagents. The colour reaction was allowed to develop until the positive control was a strong blue colour then stopped with 50 µl/well 2.5 M H₂SO₄. Data directly comparing ligands coated on an equimolar basis was calculated as percentage of the positive control (A-domain binding to native type I collagen), to take into account the slight variability in colour development.

For experiments carried out at 37°C, type I collagen and the fragments were coated and blocked at room temperature prior to assay at 37°C. In some experiments, type I collagen fragments were incubated for 16 hours at 35°C prior to coating to ELISA plates to ensure that they were completely denatured. Denatured fragments were coated directly following denaturation alongside native type I collagen for 1 hour at 37°C. BSA (50 mg/ml) in TBS was used to block unbound plastic for 1 hour at 37°C and wells were washed with TBS, 1 mM MgCl₂ as before. Both the block and wash buffer were warmed to 37°C prior to use. Biotinylated A-domain was diluted in warmed buffer and added to wells with and without antibodies or EDTA for 30 minutes at 37°C. This optimized time point was determined by time course assay (data not shown).

Unbound A-domain was flicked off and wells washed with warmed buffer. Wells were incubated with streptavidin-horseradish peroxidase (Amersham Life Science) in warmed buffer for 20 minutes at 37°C, washed as before and the bound streptavidin visualised as described above.

Since it was noted that mixing of the A-domain solution and EDTA a few minutes prior to addition to the assay resulted in a decrease in the EDTA-mediated inhibition of A-domain binding to type I collagen (data not shown), all reagents were added immediately to the assay upon mixture. Optimal antibody^{blocking} concentrations were determined by serial dilution (data not shown) and concentrations which gave maximal inhibition of binding using blocking antibodies and minimal non-specific inhibition using a control IgG were used in all subsequent experiments.

Results

Biotinylated, recombinant human α_2 integrin A-domain was analysed by 7% SDS PAGE and silver stained to confirm that the protein was intact (fig.4.1). Biotinylated α_2 integrin A-domain bound to type I collagen in a dose-dependent manner (fig.4.2) The optimum A-domain concentration for these experiments was determined to be 0.5 $\mu\text{g/ml}$, and all subsequent assays were carried out at this concentration. When A-domain binding to the native type I fragments was compared to A-domain binding to native type I collagen at room temperature, it was found that essentially the A-domain bound equally well to native type I collagen and the native 3/4 fragment (fig.4.3). No A-domain binding to the native 1/4 fragment of collagen was observed, even at the highest ligand concentration tested. A-domain binding to native collagen and the 3/4 fragment was completely ablated by addition of 10 mM EDTA, suggesting that the binding was likely to be of the same specific nature as binding of the intact integrin to collagen and the 3/4 fragment (fig.4.3).

A-domain binding to native collagen and the 3/4 fragment was inhibited by inclusion of inhibitory 6F1 or 5E8 anti- α_2 integrin antibodies as well as EDTA. Inclusion of a non-specific IgG control antibody had significantly less effect on A-domain binding to native collagen or fragments at room temperature (fig.4.4), confirming the specificity of $\alpha_2\beta_1$ integrin A-domain interactions with native type I collagen and the 3/4 fragment.

Denaturation of the collagen fragments completely ablated A-domain binding to the 3/4 fragment when assayed at 37°C, although this temperature was permissive for A-domain binding to native type I collagen (fig.4.5). This result suggests that the α_2 integrin A-domain is responsible for recognition of the triple-helical form of the type I collagen 3/4 fragment by the whole $\alpha_2\beta_1$ integrin, but that denaturation of the fragments prohibits their interaction with α_2 integrin A-domain and therefore the whole integrin molecule.

Discussion

These data confirm that monomeric type I collagen is a ligand for the α_2 integrin A-domain, and show that the A-domain binding regions are located within the N-terminal, 3/4 fragment of triple-helical type I collagen. The inability of the α_2 integrin A-domain to bind to the heat-denatured collagen fragments confirms that collagen triple-helical conformation is essential for support of $\alpha_2\beta_1$ integrin binding.

These data are in agreement with and extend previous studies of α_2 integrin A-domain-type I collagen interactions. Recombinant human α_2 integrin A-domain bound strongly to native monomeric type I collagen at room temperature and 37°C. Interestingly, although very little binding to the 1/4 fragment was observed, the A-domain was able to interact with the native 3/4 fragment of type I collagen at room temperature. A-domain binding to the 3/4 fragment at room temperature was as strong as to native collagen. This suggests that all the $\alpha_2\beta_1$ integrin A-domain binding sites are located within the

3/4 fragment of type I collagen, but that the C-terminal quarter of the molecule contains none of these binding sites.

Denaturation of the collagen fragments resulted in total abrogation of A-domain binding to both the 3/4 and the 1/4 fragments, although A-domain binding to native collagen was still possible at this temperature. These results clearly demonstrated the necessity of the collagen triple-helical conformation to support α_2 integrin A-domain binding to type I collagen and its cleavage products. In addition, these data confirmed results obtained studying whole $\alpha_2\beta_1$ integrin interactions with the same substrates, are supported by published data concerning the importance of collagen CNBr fragment conformation for $\alpha_2\beta_1$ integrin-mediated platelet adhesion (Morton et al., 1994). Collagenase degradation of type I collagen is likely to prevent $\alpha_2\beta_1$ integrin binding by generating collagen fragments which denature at physiological temperature, thereby altering cell attachment to a type I collagen substrate.

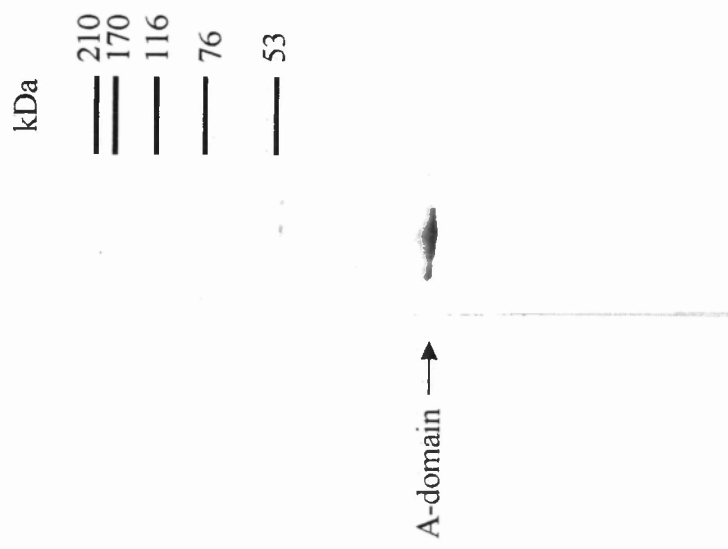


Fig.4.1: Silver stain of the purified α_2 integrin A-domain. The marked band of protein corresponds to the A-domain.

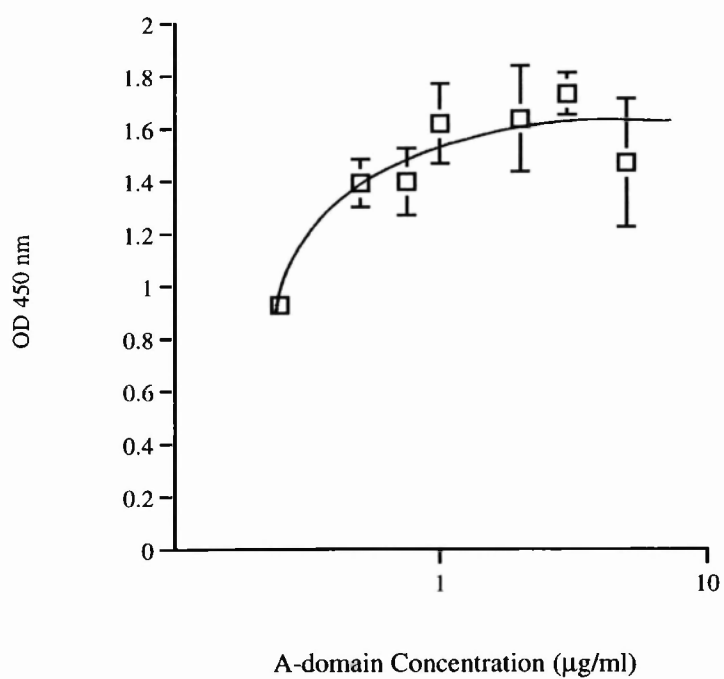


Fig.4.2 : Biotinylated α_2 integrin A-domain binding to immobilized type I collagen increased with increasing A-domain concentration. Values are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are smaller than the point markers.

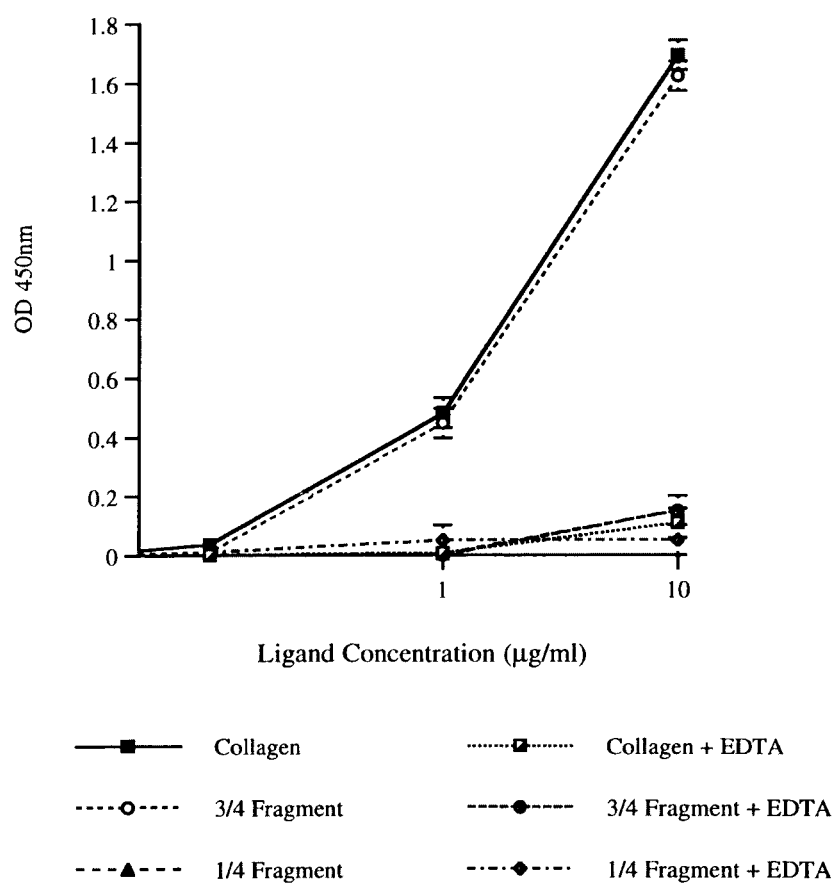


Fig.4.3 : Biotinylated α_2 integrin A-domain bound to native type I collagen and the 3/4 fragment of collagen equally well. No binding to the 1/4 fragment was observed. EDTA (10 mM) completely inhibited A-domain binding to collagen and the 3/4 fragment. Values shown are the mean of triplicates, +/- standard deviation. Where no error bars are visible, bars are smaller than the point markers.

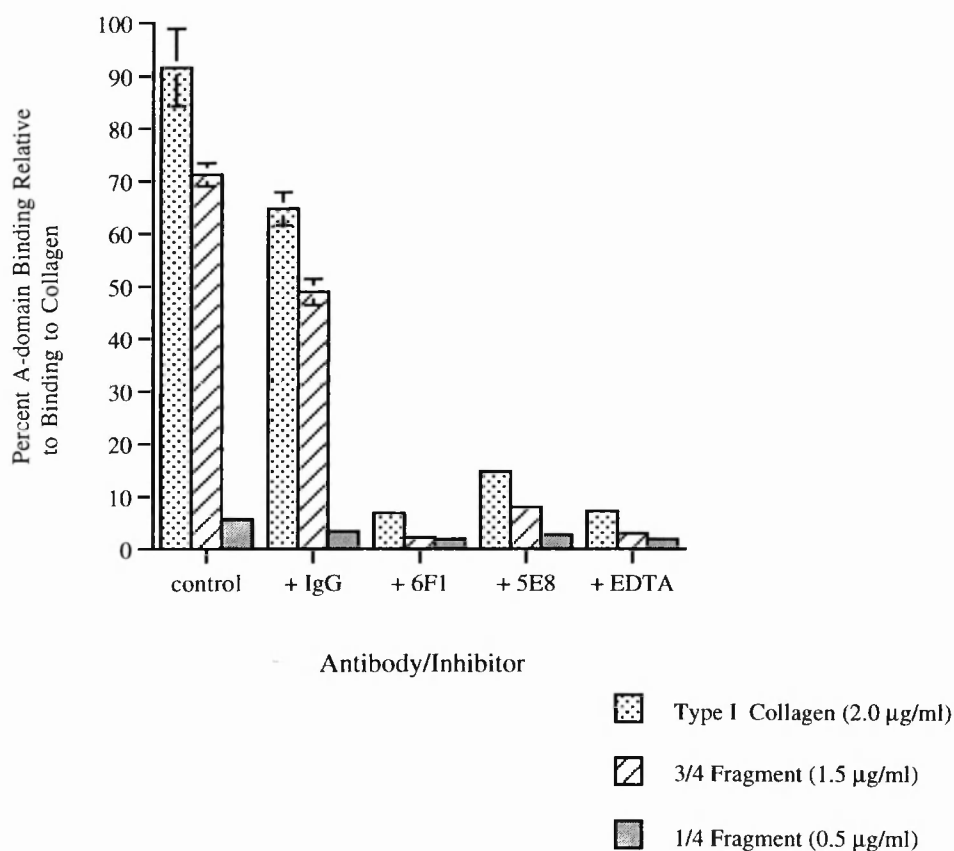


Fig.4.4 : Biotinylated A-domain binding to type I collagen and collagen fragments at room temperature was inhibited by addition of 6F1 and 5E8 anti- α_2 integrin antibodies (5.0 µg/ml) and 10 mM EDTA. Mouse IgG had no effect on A-domain binding. Values denoted as "control" refer to A-domain binding to collagen and the fragments in the absence of inhibitors. Collagen and the fragments were coated on an equimolar basis for direct comparison. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are too small to be visible on this scale.

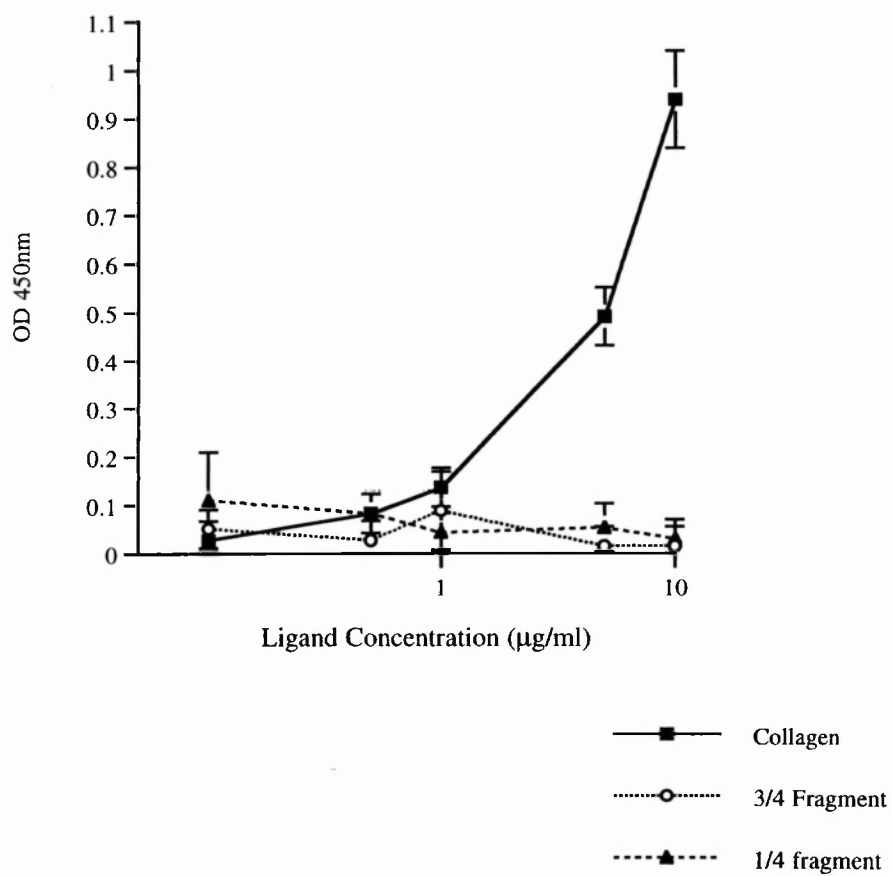


Fig.4.5 : Denaturation of the 3/4 fragment of type I collagen completely ablated biotinylated α_2 -integrin A-domain binding. A-domain binding to native collagen was still possible at 37°C. Values shown are the mean of triplicates, +/- standard deviation.

Chapter 5

Cell Attachment to Type I Collagen and CL-3 Derived Fragments

Introduction

Studies using isolated $\alpha_2\beta_1$ integrin, and α_2 integrin A-domain are useful to determine the ability of collagenase-generated type I collagen fragments to support receptor binding at, or below, physiological temperature. However, the information to be gained from this type of experiment is limited because integrins have been shown to do more than simply anchor cells to the ECM. As has been described in Chapter 1, integrins are signaling molecules which transduce both "outside-in" and "inside-out" signals. These signals can lead to major changes in cell activity and are crucial to maintaining dynamic interactions between a cell and its extracellular environment.

From the results of the solid-phase ligand binding assays using purified $\alpha_2\beta_1$ integrin, and its A-domain, collagenase cleavage of type I collagen might be predicted to lead to a significant reduction in $\alpha_2\beta_1$ integrin-mediated cell attachment to the denatured fragments. HT1080 cells express a single fibrillar collagen receptor, $\alpha_2\beta_1$ integrin (Grenz et al., 1993), and antibodies such as 6F1 (Coller et al., 1989) and 5E8 (Chen et al., 1991), which are specific to the α_2 integrin subunit, can completely inhibit cell attachment to type I collagen via this integrin.

A375M cells express low levels of $\alpha_2\beta_1$ integrin and also an RGD-dependent integrin, $\alpha_v\beta_3$ (Marshall et al., 1991), which has been shown to be a receptor for denatured collagen on some cell types (Davis, 1992; Montgomery et al., 1994). RGD-dependent integrin adhesion can be blocked by function-blocking antibodies directed at the

individual integrins or by short, RGD-containing peptides which bind to the integrins and compete with the normal substrate.

In this chapter, the ability of HT1080 cells and A375M cells to attach to native, triple-helical type I collagen and the 3/4 and 1/4 fragments was studied, and the integrins responsible for cell attachment to each substrate determined using function-blocking anti-integrin antibodies and peptide ECM-ligand mimics. Cell interactions with heat-denatured type I collagen fragments were also examined, to begin to elucidate possible *in vivo* effects of collagenase degradation of type I collagen on cell-collagen interactions.

Methods

Cell Culture

HT1080 K2P fibrosarcoma cells and A375M melanoma cells were obtained and maintained as described in Chapter 2. Cells were passaged 24-48 hours prior to experimentation and harvested at 80% confluency using trypsin/EDTA solution (Sigma). Cells were suspended in serum-free DMEM and centrifuged at 1500 g for 5-10 minutes to remove the trypsin:EDTA solution. The cell pellet was resuspended in serum free DMEM, cells counted using a haemocytometer and diluted in DMEM to the desired concentration.

Antibodies, Peptides and Substrates

Mouse monoclonal antibodies to human α_2 integrin (6F1 and 5E8), $\alpha_v\beta_3$ integrin (LM609) and mouse IgG were obtained as described in Chapter 2. All antibodies were used at 2.5-5.0 $\mu\text{g/ml}$. GRGDS and GRYGS peptides were purchased from Sigma and

used at 20 µg/ml. Rat skin type I collagen and purified type I collagen fragments were produced as described in Chapter 2. The mixture of 3/4 and 1/4 collagen fragments produced by collagenase cleavage was initially used as a substrate for cell attachment and is referred to as "cleaved collagen".

Attachment Assays

Since type I collagen fragments denature below physiological temperature, it was appropriate to test their adhesive capacity before and after denaturation. Attachment assays were performed essentially as by Gamble et al. (1993) with some modifications. Type I collagen, gelatin, cleaved collagen fragment mixture and isolated collagen fragments in TBS were plated onto 96 well tissue culture plates (Corning) for 1 hour at room temperature or 16 hours at 4°C and blocked with 1% heat-denatured BSA in TBS (Sigma) for 1 hour at room temperature or 2 hours at 4°C. Where it was necessary to prevent renaturation, these steps were performed at 37°C with warmed reagents. Wells were washed with tissue culture medium and cells plated for the required time at the desired temperature. Unattached cells were removed at the end of the assay by a sharp flicking motion (HT1080 cells) or by draining (A375M cells). Wells were washed three times with tissue culture medium and attached cells fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. Fixed cells were stained for 30 minutes at room temperature with 1% methylene blue in 0.01% sodium tetraborate, washed thoroughly under running water and lysed with ethanol/1M HCl (50:50 mixture). Released dye was gently mixed and the colour intensity measured by spectrophotometer at 630 nm wavelength.

Cell Attachment at Room Temperature

In order to assess cell attachment at room temperature (approximately 21°C), cells were harvested in DMEM, washed in an air buffered tissue culture medium (L-15; Sigma,

USA) and resuspended in L-15 medium. Cells were plated onto type I collagen in 100 μ l of L-15 medium at 6×10^5 cells/ml for up to 2 hours at room temperature. The optimal time for assay at this temperature was determined to be between 1 and 1.5 hours. Where antibodies were used, cells were incubated with these for 30 minutes in L-15 medium at room temperature prior to use in the assay.

Cell Attachment at 37°C

Cell attachment to type I collagen and fragments was tested at physiological temperature in two ways. Adsorption of gelatin, cleaved collagen and isolated fragments to tissue culture plastic at room temperature appeared to stabilize the substrates and encourage adoption of the triple helical conformation by the denatured substrates. Therefore, collagen, gelatin, cleaved collagen and fragments were adsorbed to 96 well plates at room temperature and 37°C, to allow comparison of cell attachment to native and denatured substrates at physiological temperature. Collagen fragments were denatured by incubation at 35°C for 16 hours. This temperature was chosen because it is above the fragment's melting temperature but below the melting temperature of monomeric type I collagen. Collagen was denatured to gelatin by heating to 60°C for 20 minutes. To ensure neither the fragments or gelatin had opportunity to renature during the adsorption process, these were coated onto warmed 96 well plates at 37°C. Blocking and washing stages were also performed at 37°C using warmed reagents. Cells were harvested, washed and resuspended in DMEM and plated in 100 μ l of DMEM at 6×10^5 cells/ml for up to 2 hours at 37°C. Optimal attachment was found to occur between 30 minutes and 1 hour at this temperature. Where antibodies were used, cells were incubated with these for 30 minutes in DMEM at 37°C prior to use in the assay.

Immunolocalization of Integrins

HT1080 cells were sparsely seeded onto glass coverslips which had been coated by adsorption of type I collagen and collagen fragments for 16 hours at 4°C. Cells were cultured in DMEM in the presence of 10% FCS for 48 hours prior to washing with PBS. Cells were washed 3 times for 5 minutes each time and then fixed with 4% paraformaldehyde in PBS, pH7.4 for 5 minutes. Cells were washed as before and incubated with anti-integrin antibodies (5.0 µg/ml) in PBS, in the presence of 0.5% serum (species dependent on the species of the secondary antibodies), for 2 hours at room temperature. The primary antibody solution was removed and cells washed three times as above. Cells were incubated with immunofluorescence-conjugated secondary antibodies for 1 hour at room temperature and then washed as before. The coverslips were mounted cell-side-down onto glass slides and sealed. Cell-surface staining was observed using an immunofluorescence microscope (Zeiss) at x600 magnification. Photographs were taken using Panther colour film (Kodak) and processed at 1600 ASA.

Results

HT1080 Cell Attachment at Room Temperature

The duration of the assay at room temperature and the collagen concentration was optimised by time course and serial dilution of the substrate (fig.5.1a and 5.1b). When HT1080 cell attachment to native collagen and the cleavage mixture of fragments was compared, it was found that collagenase cleavage had made no difference to the ability of cells to attach to collagen (fig.5.2). Attachment was mediated by $\alpha_2\beta_1$ integrin, as 6F1 anti- α_2 integrin antibody greatly reduced cell attachment to native or cleaved collagen (fig.5.3).

A375M Cell Attachment at Room Temperature

Assay duration was determined by time course (fig.5.4a) and optimum collagen concentration was determined by concentration curve (fig.5.4b). No difference in attachment was observed when cells were plated onto native collagen or the cleavage mixture of fragments when these were plated at room temperature (fig.5.5).

HT1080 Cell Attachment at 37°C (adsorption at room temperature)

The optimal duration of the assay at 37°C was estimated to be between 30-60 minutes (Gamble et al., 1993). Optimum collagen concentration was determined by concentration curve (fig.5.6). Reduced HT1080 cell attachment to low concentrations of the cleavage mixture of collagen fragments, compared to attachment to native collagen at the same concentrations was observed (fig.5.7). When HT1080 cell interactions to isolated collagen fragments was analysed, it was found that these cells were only utilising binding sites within the 3/4 fragment (fig.5.8), and attachment to the isolated 3/4 fragment closely mirrored attachment to the cleavage mixture and gelatinized collagen. HT1080 cell attachment to native collagen and the 3/4 fragment could be inhibited by anti- α_2 integrin antibodies but not by a control IgG, an RGD-containing peptide or its control RYD-containing peptide (fig.5.9).

HT1080 and A375M Cell Attachment at 37°C (adsorption at 37°C)

Serial dilutions of native type I collagen and denatured fragments showed that heat-denaturation of the fragments completely ablated HT1080 cell attachment to the 3/4 fragment (fig.5.10) and gelatin (fig.5.11).

HT1080 cell attachment to native collagen could still be inhibited by preincubation of cells with 6F1 anti- α_2 integrin antibody, and occasional low levels of cell attachment to gelatin could also be completely inhibited by 6F1 (fig.5.12). As a control for collagen, gelatin and fragment adsorption to the tissue culture plastic, A375M melanoma cell

attachment was also assayed under these conditions. These cells adhered equally well to gelatin and the denatured fragments, although slightly more attachment to native collagen was observed. A375M cell attachment to collagen could be inhibited by incubation with 6F1 anti- α_2 integrin antibody, however, attachment to gelatin and the fragments was inhibited by LM609 anti- $\alpha_v\beta_3$ integrin antibody. 6F1 had little effect on A375M attachment to gelatin or denatured fragments and LM609 had little effect on cell attachment to native collagen (fig.5.13).

Immunolocalization of Integrins

The integrin profile of HT1080 cells grown on native type I collagen and type I collagen fragments was compared. HT1080 cells expressed high levels of $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrin when grown on all three substrates (fig.5.14a and b). Virtually no $\alpha_v\beta_3$ integrin could be detected by immunofluorescent staining using LM609 anti- $\alpha_v\beta_3$ integrin antibody on cells grown on any of the substrates (fig.5.14a). The suitability of LM609 for use in immunolocalization studies was confirmed by the bright staining of $\alpha_v\beta_3$ integrin on the surface of A375M cells (see Chapter 8). These results suggest that HT1080 cells may not modulate their integrin repertoire in response to the triple-helical fragments, however, the effects of denaturation of the collagen fragments on HT1080 cell integrin profile was not tested.

Discussion

Two cell surface receptors for type I collagen have been identified and confirmed to date, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins. HT1080 cells express $\alpha_2\beta_1$ integrin but not $\alpha_1\beta_1$ integrin, therefore all cell attachment to type I collagen was likely to be mediated by this integrin. The $\alpha_2\beta_1$ integrin binding sites on native type I collagen are reversibly destroyed upon unwinding of the collagen triple helix but cooling to physiological temperature allows renaturation of the triple helix (Stark and Kühn, 1968a) and

restoration of $\alpha_2\beta_1$ integrin binding sites (Morton et al., 1994). Since the 3/4 and 1/4 collagen fragments denature below 37°C, it was unlikely that these would be able to support cell attachment via $\alpha_2\beta_1$ integrin if maintained in the denatured conformation. However, it is interesting to note that if the 3/4 fragment is adsorbed to tissue culture plastic at room temperature prior to assay at 37°C, association with the plastic appears to stabilize the triple-helical conformation sufficiently to allow $\alpha_2\beta_1$ integrin-mediated cell attachment. The data confirms the hypothesis that triple-helical collagen conformation is essential for $\alpha_2\beta_1$ integrin-mediated cell attachment but also demonstrates that in native collagen conformation, all $\alpha_2\beta_1$ integrin-mediated binding sites are within the 3/4 N-terminal portion of the molecule. The data supports published observations using cyanogen bromide (CNBr) fragments of type I collagen, showing that $\alpha_2\beta_1$ integrin-mediated attachment of human platelets occurs to native CNBr fragments derived from the N-terminal of the collagen molecule and that heat-denaturation of the fragments containing binding sites ablates platelet adhesion (Morton et al., 1994).

$\alpha_v\beta_3$ integrin has been postulated to be a cryptic receptor for gelatin which is only exposed when the collagen triple helix is unwound during heat-denaturation and degraded by proteinases (Montgomery et al., 1994). HT1080 cells are not thought to express any α_v integrins (fig. 15 and K. Yamada, personal communication), so it was not unexpected that gelatinized collagen could not support HT1080 cell attachment. It was interesting to find that the heat-denatured collagen fragments were also unable to provide binding sites for HT1080 cells, suggesting that these might well provide a good model for in vivo generation of gelatin. A375M melanoma cells express both $\alpha_v\beta_3$ integrin and $\alpha_2\beta_1$ integrin, explaining why these cells adhere as well to native collagen as they do to gelatin and denatured 3/4 and 1/4 collagen fragments. LM609 anti- $\alpha_v\beta_3$ integrin antibody inhibited A375M cell attachment to the denatured substrates, lending support to the hypothesis of cryptic $\alpha_v\beta_3$ integrin-binding sites being exposed upon denaturation of type I collagen. It is very interesting to note that there is at least one of

these binding sites on both the 3/4 and the 1/4 collagen fragment as these supported A375M adhesion equally well, in contrast to $\alpha_2\beta_1$ integrin-mediated adhesion which only occurs within the 3/4 fragment.

These findings demonstrate that cleavage of type I collagen has a profound effect on cell adhesion to this substrate. Cells which are able to adhere to native, triple-helical type I collagen may not be able to form stable attachments to cleaved, denatured collagen fragments. Loss of adhesion or reduction in attachment can lead to dramatic changes in cell morphology and function, even prompting apoptotic cell death (reviewed by Ruoslahti and Reed, 1994; Metcalfe and Streuli, 1997) or tumour cell metastasis (Juliano and Varner, 1993; Varner and Cheresch, 1996). Even cells which are able to utilize another integrin receptor to remain attached to a cleaved substrate may be subjected to changes directly brought about by interactions with the modified substrate (reviewed by Sage, 1997). Integrin signaling information about the new extracellular environment may lead to alteration of cell responses to other stimuli such as growth factors (Sundberg and Rubin, 1996), thereby leading to localized disruption of an area of tissue and tumour cell invasion and metastasis.

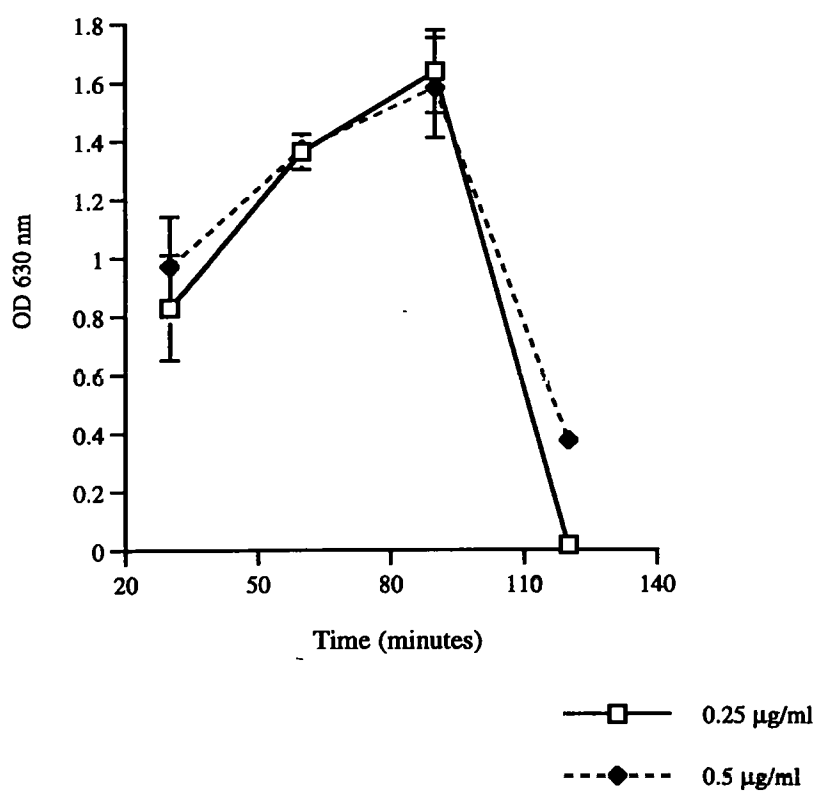


Fig.5.1a : HT1080 cell (6×10^5 cells/ml) attachment to native type I collagen at room temperature at 0.5 µg/ml and 0.25 µg/ml collagen concentration increased with time up to 90 minutes after plating, but decreased after this time. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are smaller than the point markers.

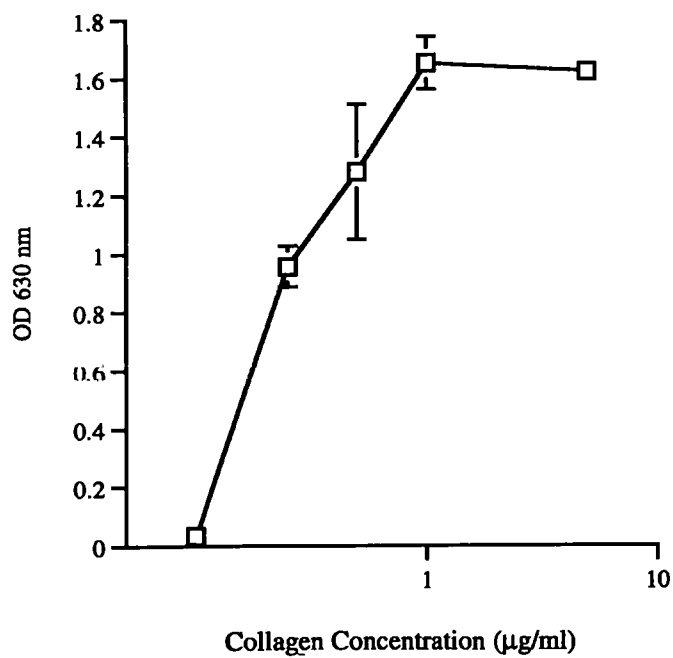


Fig.5.1b : HT1080 cell attachment to native type I collagen at room temperature increased with increasing collagen concentration over 1 hour. Values shown are the mean of triplicates \pm standard deviation. Where error bars are not shown, bars are smaller than the point markers.

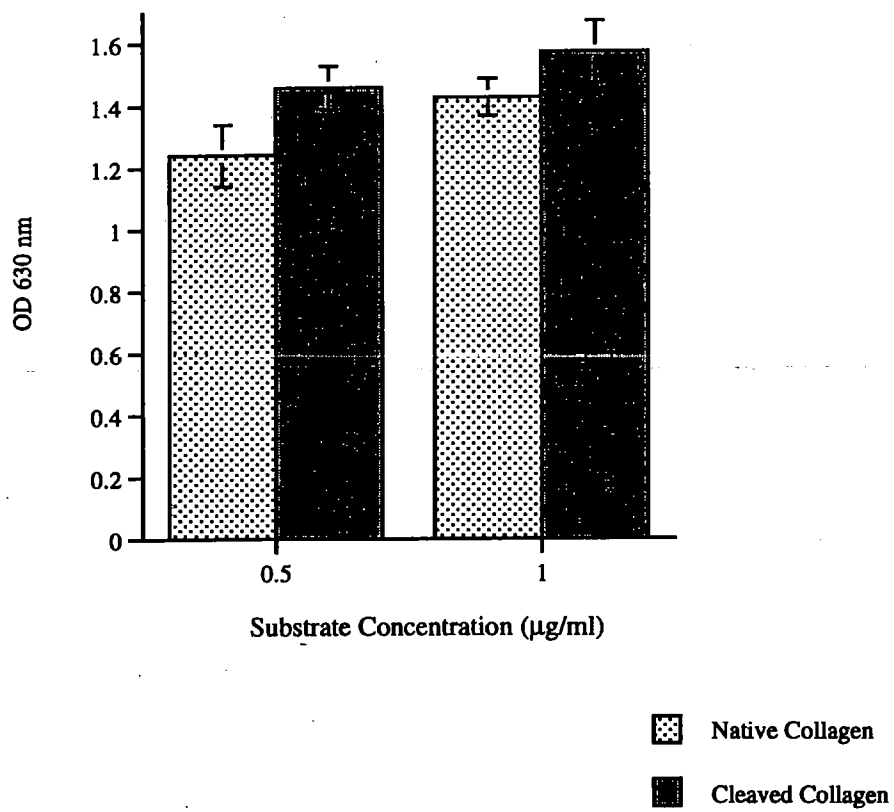


Fig.2 : Collagenase-3 cleavage of type I collagen had no effect on HT1080 cell (6×10^5 cells/ml) attachment at room temperature. Native and cleaved collagen were plated and assayed at room temperature for 1.5 hours at 1.0 and 0.5 µg/ml. Values shown are the mean of triplicates, +/- standard deviation.

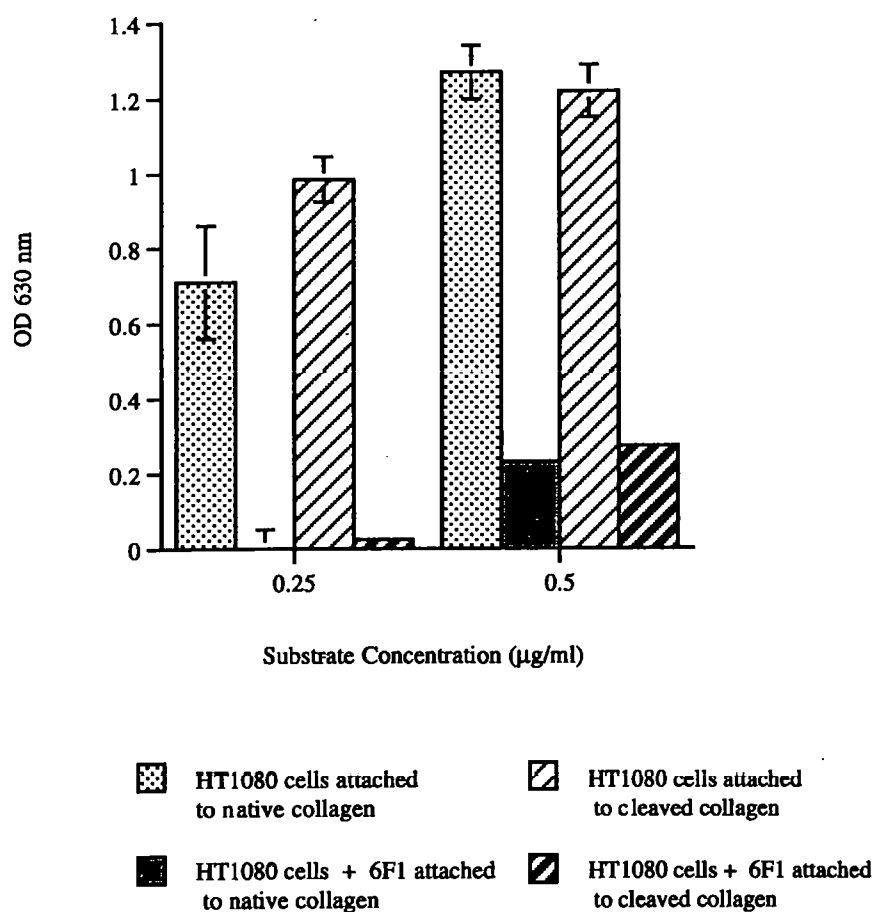


Fig.5.3 : HT1080 cell (6×10^5 cells/ml) attachment to native and cleaved collagen coated and assayed at room temperature at 0.5 and 0.25 µg/ml, was inhibited by 6F1 anti- α_2 integrin antibody (2.5 µg/ml). Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are too small to be visible on this scale.

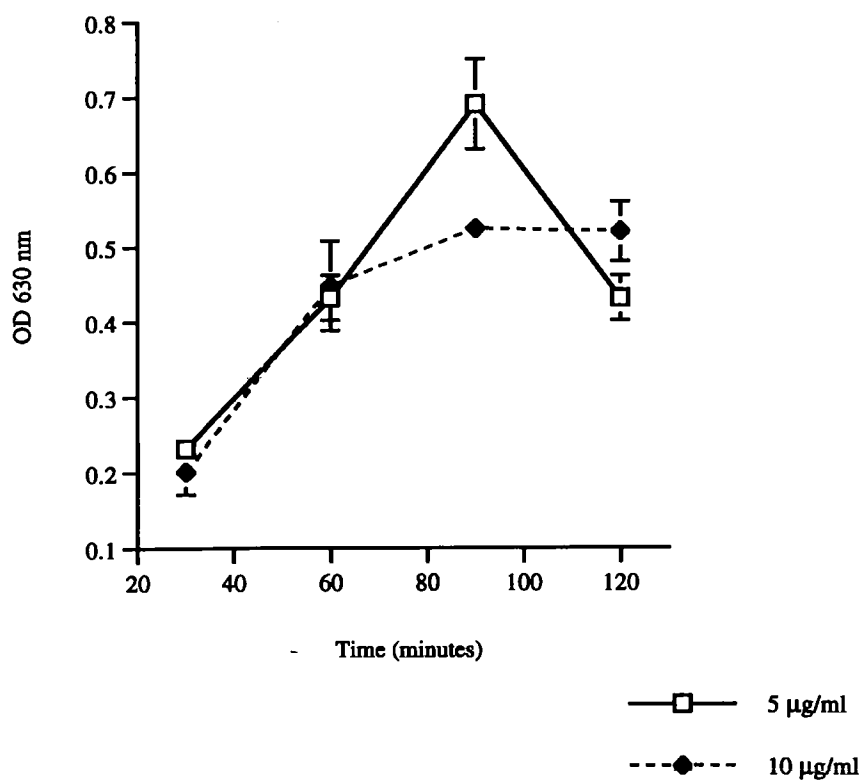


Fig.5.4a : A375M cell attachment at room temperature to type I collagen plated at 10.0 and 5.0 µg/ml reached maximum at approximately 90 minutes after plating. No further increase in cell attachment was observed after this time. Values shown are the mean of triplicates, +/- standard deviation.

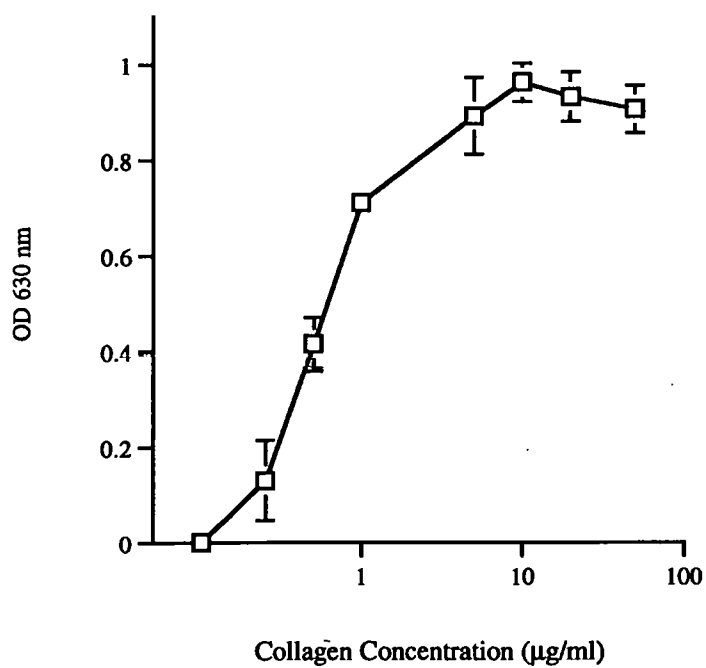


Fig.5.4b : A375M cell (6×10^5 cells/ml) attachment at room temperature to type I collagen increased with increasing collagen concentration. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are smaller than the point markers.

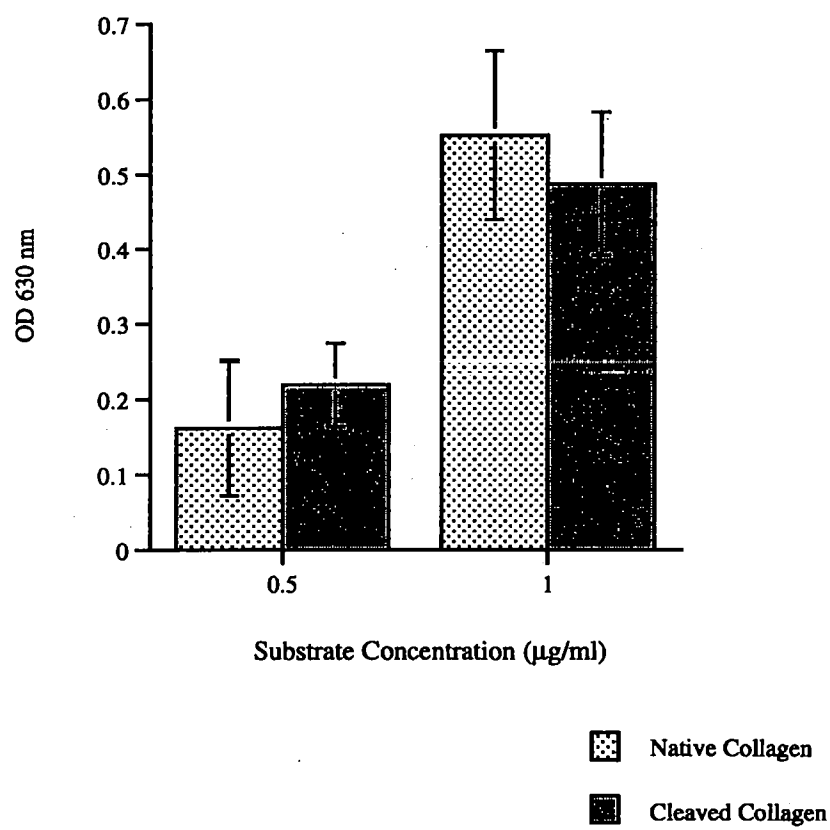


Fig.5.5 : Collagenase-3 cleavage of type I collagen had no effect on A375M cell attachment to collagen plated and assayed at room temperature at 1.0 and 0.5 µg/ml. Values shown are the mean of triplicates, +/- standard deviation.

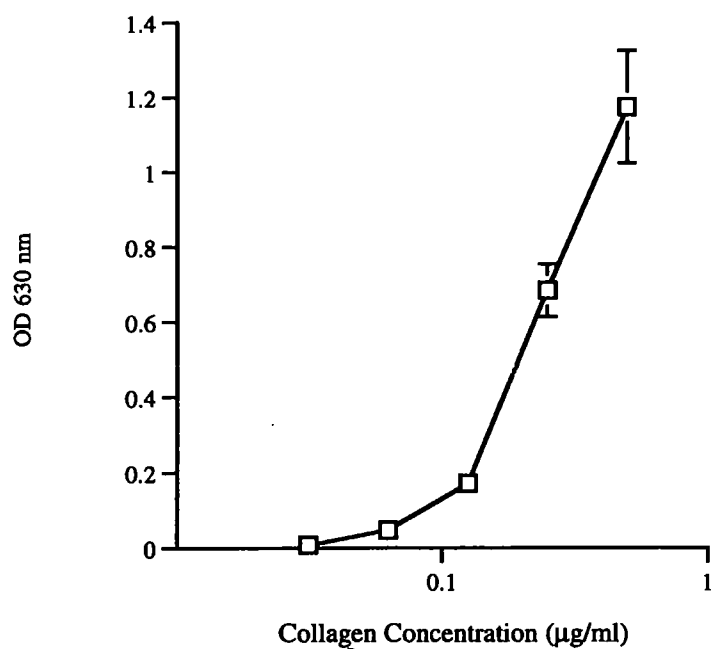


Fig.5.6 : HT1080 cell attachment to collagen at 37°C for 1 hour increased with increasing collagen concentration. Values shown are the mean of triplicates, +/- standard deviation. Where error bars are not shown, bars are smaller than the point markers.

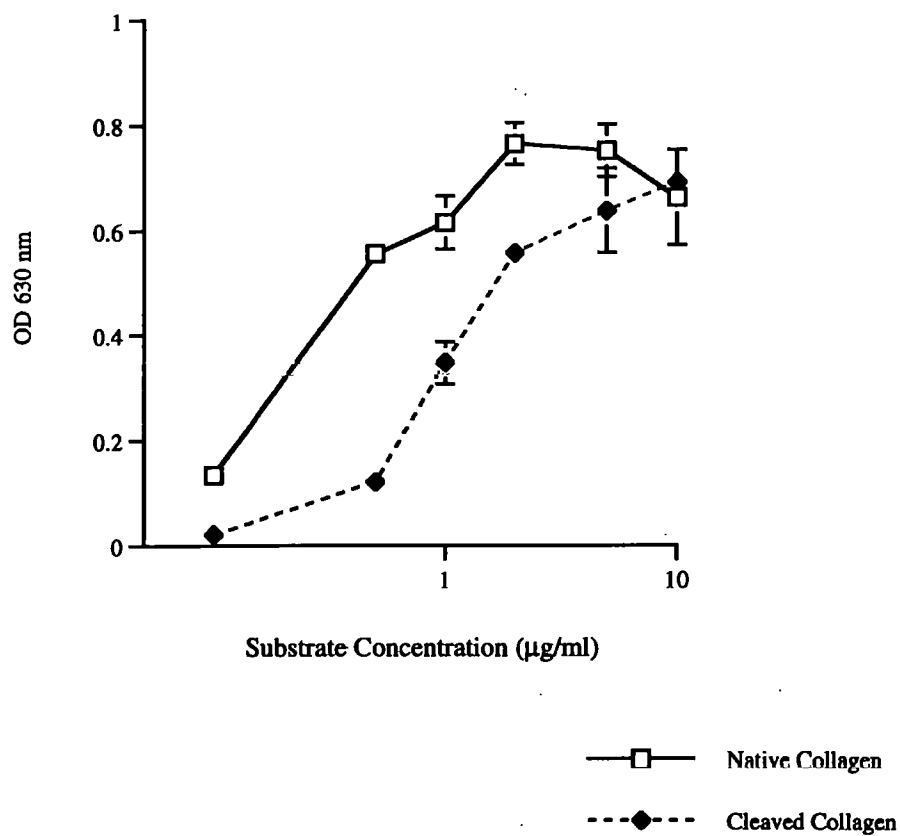


Fig.5.7 : Attachment of HT1080 cells (6×10^5 cells/ml) to cleaved type I collagen was generally lower than cell attachment to native collagen. Substrates were plated at room temperature and cell attachment assayed for 1 hour at 37°C . Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are smaller than the point markers.

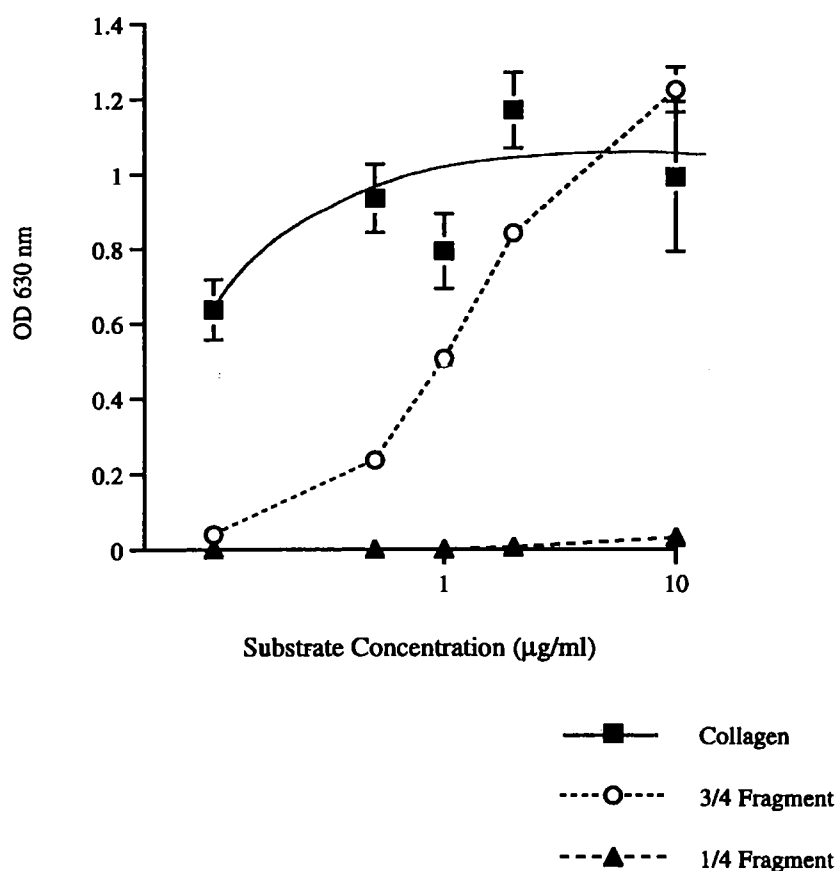


Fig.5.8 : HT1080 cell (6×10^5 cells/ml) attachment to native type I collagen was already near maximal at 0.1 μg/ml collagen coating concentration, however, at least 1.0 μg/ml coating concentration of 3/4 fragment was required to reach half maximal cell attachment. Virtually no cell attachment to the 1/4 fragment was observed. Substrates were plated at room temperature and cell attachment assayed for 1 hour at 37 °C. Values shown are the mean of triplicates, +/- standard deviation. Where error bars are not shown, bars are smaller than the point markers.

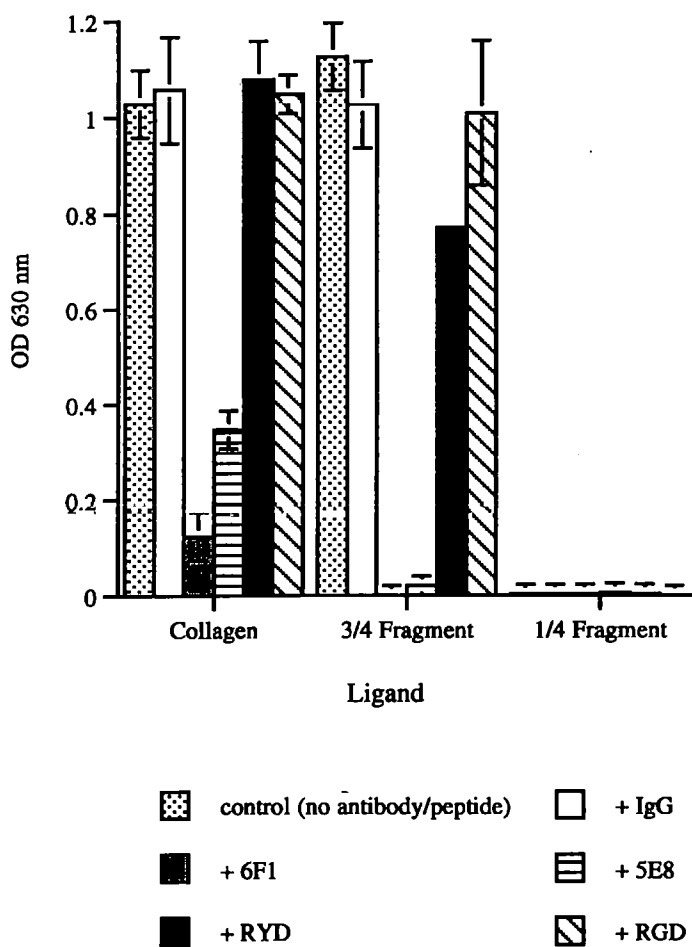


Fig.5.9 : HT1080 cell (6×10^5 cells/ml) attachment to native collagen and the isolated 3/4 collagen fragment at 37°C was inhibited by 6F1 and 5E8 anti- α_2 integrin antibodies but not by control IgG or RGD/RYD peptides. No cell attachment to the 1/4 fragment was observed at this concentration ($0.5 \mu\text{g/ml}$). Collagen and fragments were compared on an equimolar basis. Antibodies used at $5.0 \mu\text{g/ml}$, peptides used at $20.0 \mu\text{g/ml}$. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are too small to visualize on this scale.

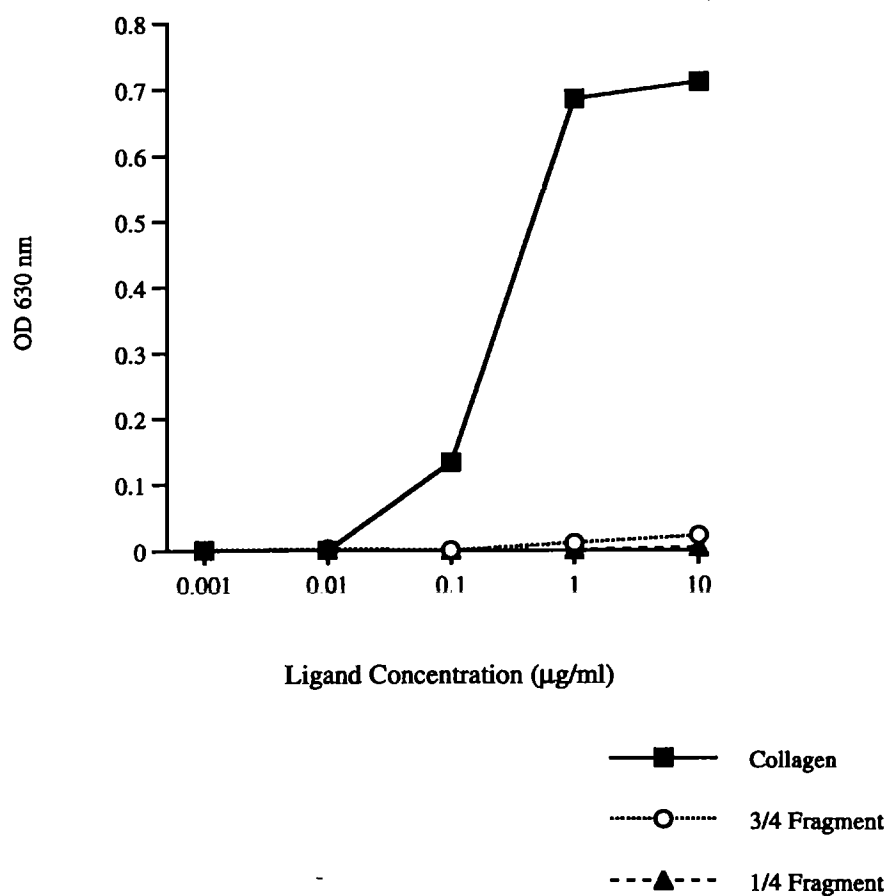


Fig.5.10 : HT1080 cell (6×10^5 /ml) attachment to type I collagen increased with increasing collagen concentration, however, cells were not able to attach to either denatured collagen fragment even at high fragment coating concentrations. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are smaller than the point markers.

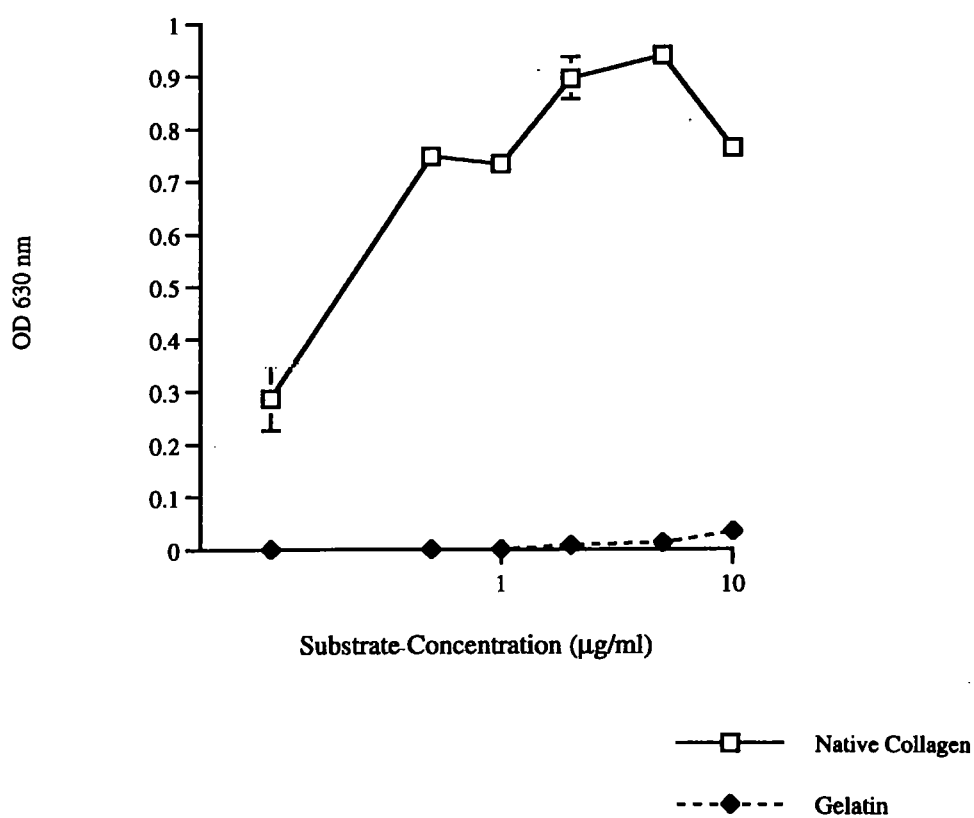


Fig.5.11 : HT1080 cell (6×10^5 cells/ml) attachment to native collagen increased with increasing collagen concentration, however, cells were not able to attach to gelatin (heat-denatured collagen), even at high coating concentrations. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are smaller than the point markers.

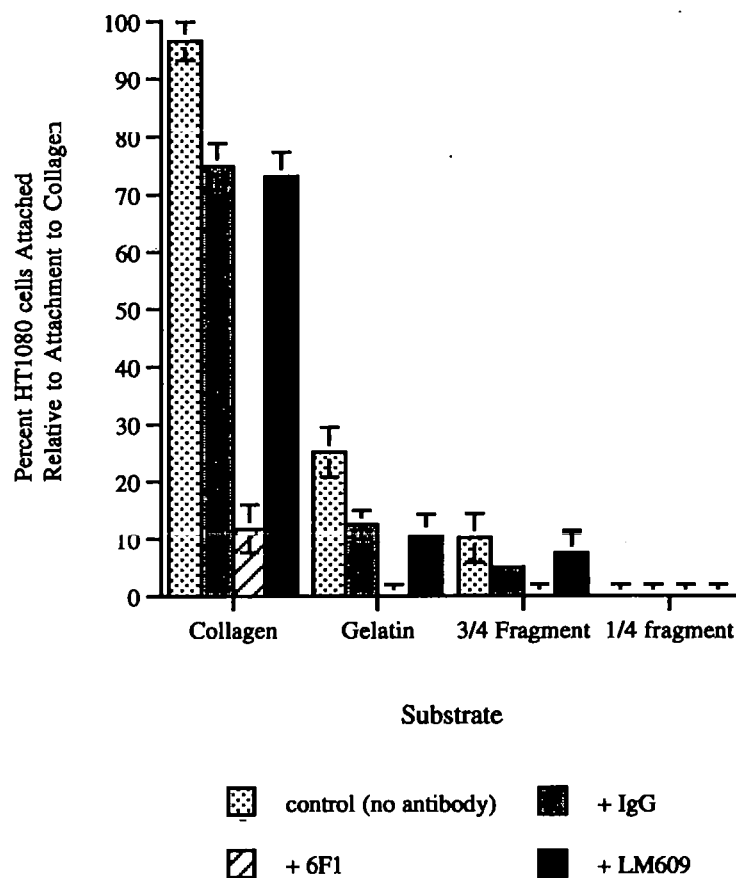


Fig.5.12 : HT1080 cell attachment to type I collagen was inhibited by 6F1 anti- α_2 integrin antibody but not by LM609 anti- $\alpha_v\beta_3$ antibody or control IgG. Residual HT1080 attachment to gelatin and the denatured 3/4 fragment were also inhibited by 6F1. No cells attached to the denatured 1/4 fragment. All substrates were used at 10.0 $\mu\text{g/ml}$, all antibodies used at 5.0 $\mu\text{g/ml}$. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are too small to be visualized on this scale.

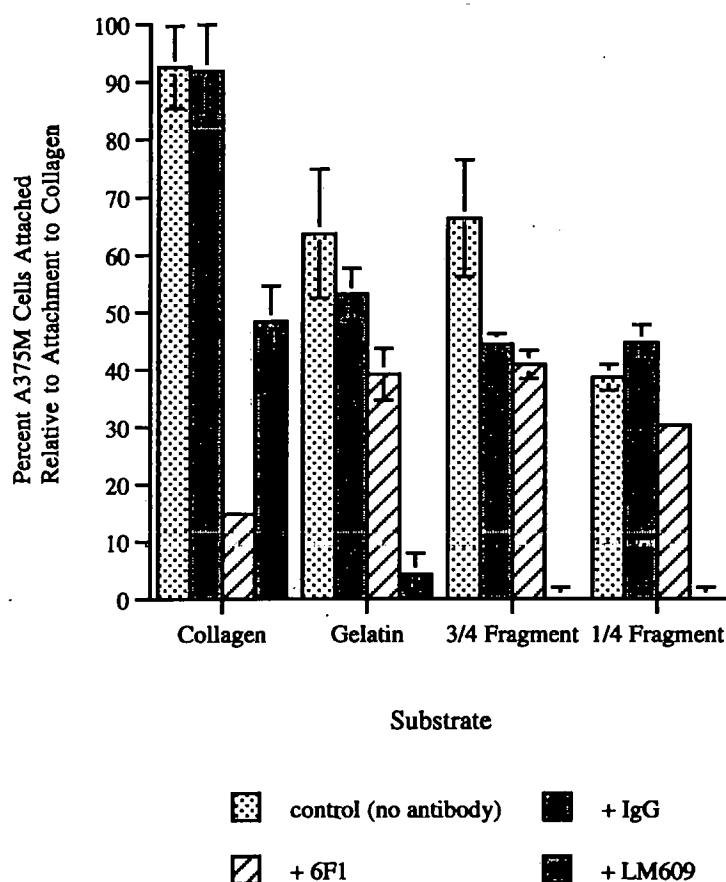


Fig.5.13 : A375M attachment to type I collagen was inhibited by 6F1 anti- α_2 integrin antibody but not by LM609 anti- $\alpha_v\beta_3$ integrin antibody. In contrast, A375M cell attachment to gelatin and the denatured collagen fragments was inhibited by LM609 but not by 6F1. A control IgG had little effect on A375M cell attachment to the substrates. All substrates were used at 10.0 $\mu\text{g/ml}$, all antibodies were used at 5.0 $\mu\text{g/ml}$. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are too small to visualize on this scale.

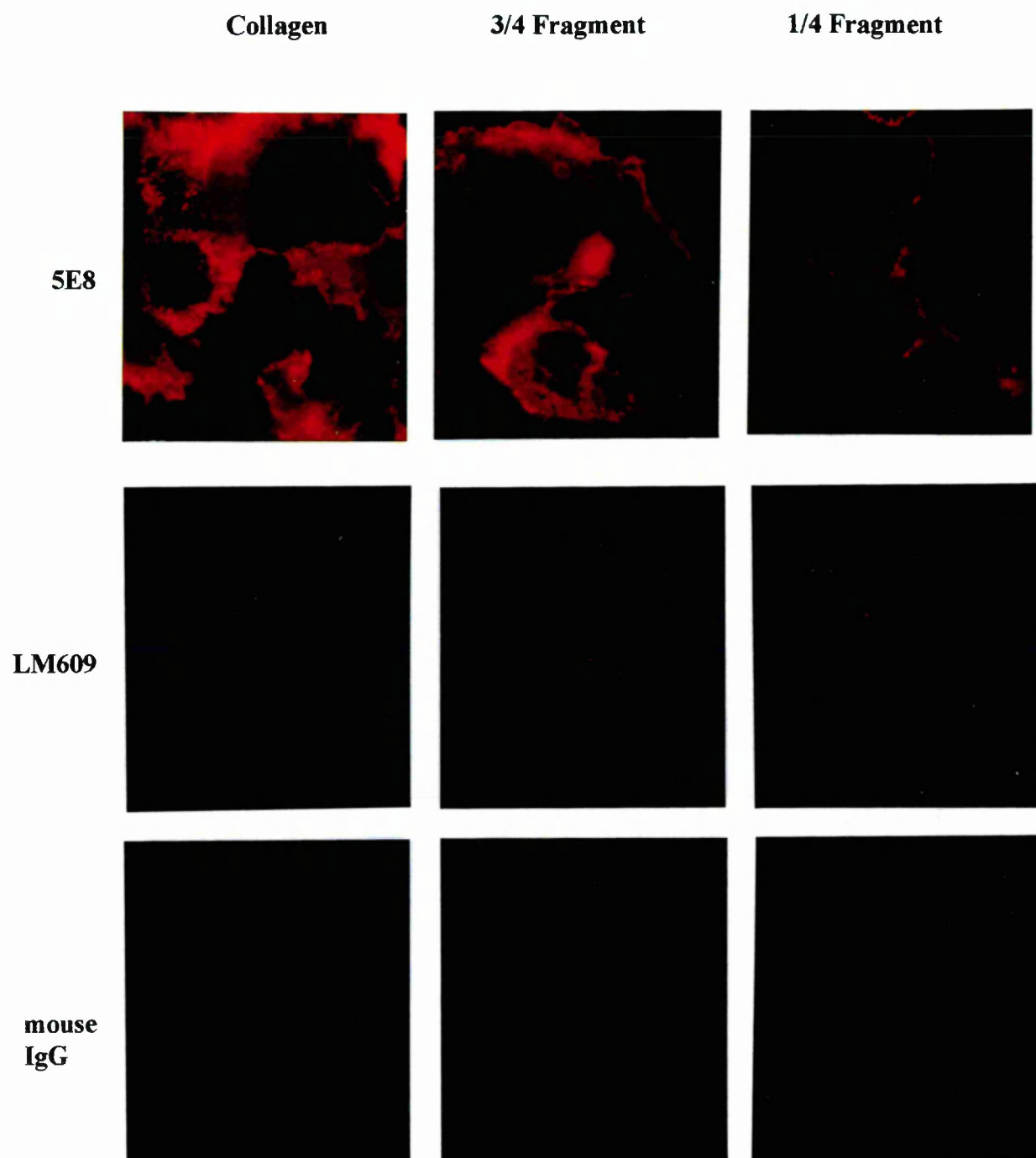


Fig.5.14a: HT1080 cells highly express $\alpha_2\beta_1$ integrin when grown on native type I collagen and the 3/4 and 1/4 collagen fragments, however, these cells apparently expressed very little $\alpha_v\beta_3$ integrin when grown on any of the collagenous substrates. 5E8 anti- α_2 integrin antibody, LM609 anti- $\alpha_v\beta_3$ integrin antibody and control mouse IgG were used at 5.0 micrograms/ml.

0 30 μm

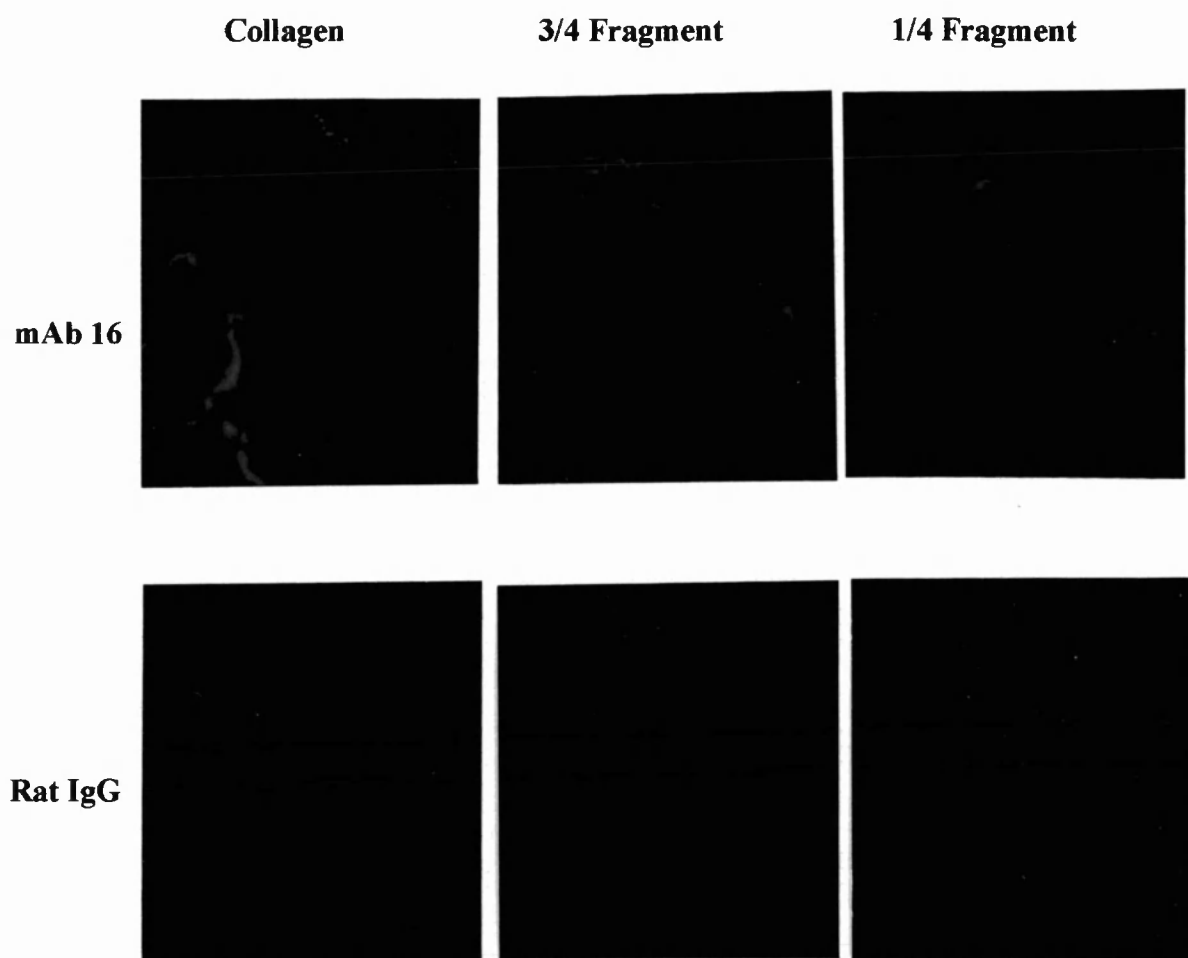


Fig.5.14b: HT1080 cells highly expressed $\alpha_5\beta_1$ integrin when grown on native type I collagen and the 3/4 and 1/4 collagen fragments. mAb 16 anti- α_5 integrin antibody and control rat IgG were used at 5.0 micrograms/ml.

0 ————— 30 μ m

Chapter 6

Cell Invasion Through Collagen and Cleaved Type I Collagen Fragments

Introduction

The role of MMPs during cell invasion through ECM proteins was studied by investigating the effects of prior *in vitro* cleavage of type I collagen on HT1080 fibrosarcoma cell invasion. To ensure that the ECM substrate was completely degraded, purified type I collagen fragments (generated by collagenase-3 cleavage of type I collagen) were coated to the filters and cell invasion was compared to invasion through native collagen. The role of $\alpha_2\beta_1$ integrin in cell invasion through these ECM proteins was also explored using function-blocking anti-integrin antibodies and RGD-containing peptides. The roles of MMPs and serine proteinases in cell motility through native and cleaved collagen were investigated with TIMP-2 and a synthetic MMP inhibitor CT1746 of metalloproteinases, as well as the serine proteinase inhibitor, aprotinin.

Methods

Reagents

Anti- α_2 integrin antibodies and a control IgG were obtained as described in Chapter 2. BSA and RGD/RVD containing peptides were purchased from Sigma as described in Chapter 2. A synthetic MMP inhibitor, CT1746, was donated by Celltech, UK. Aprotinin was purchased from Sigma.

Tissue Culture

HT1080 cells were obtained and maintained as described in Chapter 2. Cells were passaged 24-48 hours prior to experimentation and removed from tissue culture flasks using trypsin:EDTA as described in Chapter 2. Cells were suspended in serum-free DMEM and centrifuged at 1500 g for 5-10 minutes to remove the trypsin:EDTA solution and cells resuspended in DMEM. Cells were counted using a haemocytometer and diluted to the required concentration in DMEM, with or without antibodies/inhibitors.

NIH 3T3 cells were obtained from the ETACC and maintained in DMEM supplemented as described in Chapter 2.

Development of a Serum-Free Chemoattractant

NIH 3T3 conditioned medium (CM) was generated essentially as described by Fridman et al. (1990). The cells were supplemented with 5 µg/ml insulin, 0.2% lactalbumin hydrolysate (LH) and 50 µg/ml ascorbic acid for 24 hours. Ascorbic acid was later omitted. Excess CM was stored at -20°C until needed.

Gelatinases were removed from NIH 3T3 CM by batch affinity chromatography using gelatin-Agarose. Briefly, CM was swirled gently with gelatin-coupled Sepharose (prepared as instructed by the manufactures, Sigma) for 2 hours at room temperature. The mixture was centrifuged at 500 g for 2 minutes and the CM supernatant removed. The presence of residual gelatinases was checked by gelatin zymography but the purified CM was found to be gelatinase-free (data not shown). Gelatinases were removed from commercial preparations of human plasma fibronectin (FN) by passing the FN preparation over a zinc-chelated Sepharose (Sigma) column as described by Smilenov et al. (1992). Purified FN was checked for residual gelatinase presence by gelatin zymography but no evidence of pro or active gelatinases was found. Protein concentration was determined by BCA assay as described in Chapter 2.

Although a trend towards less invasion/migration can be seen during the course of the experiments using gelatinase-depleted FN as the sole chemoattractant, it was felt that the purified FN had sufficient chemotactic ability to use in the system. However, when FN was tested in the 6 hour invasion assay, an unacceptably high degree of variability in cell migration and invasion from assay to assay was recorded (data not shown). Serum was found to stimulate a consistent level of cell invasion and therefore was used during short (6 hour) assays.

The possibility of using growth factors (GFs) as chemoattractants was briefly explored. These had advantages over the CM system because the quantity of each GF was defined and was not subject to fluctuations due to the state of the conditioning cells. It was decided to focus on the chemotactic ability of scatter factor (hepatocyte growth factor), as this has proven ability to attract hepatocytes (Gherardi et al., 1989) and is produced by NIH 3T3 cells (Rong et al., 1994). This was compared to the chemotactic effect of 3T3 CM and found to be substantially less able to attract HT1080 cells than the 3T3 CM (data not shown).

Invasion Assays

The invasion system chosen, (Transwell, Costar), has been extensively used by other researchers and consists of an 8 μ m pore-sized filter at the bottom of a 0.5 ml volume disposable plastic insert (upper well) which is suspended above a 1.5 ml volume lower well (fig.1). Cells in unsupplemented culture medium are placed into the upper well which is then placed into the lower well containing tissue culture medium and a chemoattractant. The filter may be coated with ECM proteins prior to the addition of cells to provide a barrier to invasion. This system provides a simple *in vitro* model of cell invasion through ECM and is easily adapted to allow study of the effects of antibodies and exogenously added proteinases or inhibitors.

The invasion assay method was based on the protocol of Albini et al. (1987). Type I collagen was cleaved as described in Chapter 2 and air-dried onto filters for 16 hours at room temperature. Filters were washed and rehydrated as described above. Type I collagen fragments were prepared as described in Chapter 2 and air-dried onto filters either as for the cleaved collagen mixture or overnight at 37°C. Gelatin was prepared as described in Chapter 2 and air-dried onto filters for 16 hours at 37°C. Cells were removed with trypsin/EDTA, spun down in the absence of serum and resuspended in DMEM. Two $\times 10^5$ cells/well were placed in the upper well of each chamber and cells allowed to invade through the matrix towards a chemoattractant in the lower chamber for 6-18 hours at 37°C. If antibodies were to be included, cells were incubated in the presence of antibody for 30 minutes at 37°C prior to assay. Medium in upper and lower chambers was harvested and invasive cells were trypsinised off the lower chambers and the bottom of the filters, placed in DMEM+10% FCS, spun down and resuspended in DMEM+10% FCS (50-100 μ l/well). Cells were counted using a haemocytometer. Medium collected from upper and lower chambers was analysed by 8% gelatin zymography and 11% reverse gelatin zymography (see Chapter 2) to assess cells production of gelatinases and TIMPs.

Results

To avoid contaminating the 24 hour invasion system with MMPs and TIMPs it was necessary to devise a serum-free chemoattractant. Ideally, this would be tissue culture medium with defined supplements. Initially 3T3 CM was assessed because other groups (Albini et al., 1987; Fridman et al., 1990) found it to contain powerful chemoattractants, although these could not be defined. Upon gelatin zymography of the CM generated, it was found that this too contained gelatinases (data not shown) and once these were removed by gelatin agarose purification, chemotactic ability was lost.

Addition of gelatinase-free FN back to the CM restored this ability. As FN can also be purified over gelatin agarose it is likely the FN content of the CM was removed at the same time as the GLA and GLB. Although the purified CM + FN was a reasonably good chemoattractant when compared to serum, (10-50% of cells which migrated towards 10% serum would migrate towards CM + FN), there were many unknown factors also present in the CM which could also be influencing invasion/migration. However, FN proved to be a chemoattractant in its own right, migration of HT1080 cells towards DMEM+20 µg/ml gelatinase-free FN was approximately 25% of that towards DMEM+10% serum, and roughly equal to that towards CM + FN. A summary of the results obtained using the various chemoattractants tested in the development of a serum-free system is shown in Table 1.

Following purification over a zinc-chelate Sepharose column, the sample of the fibronectin was checked for gelatinase activity by gelatin zymography (method described in Chapter 2). To maximise the chance of gelatinolytic enzymes degrading the substrate the zymogram was incubated in assay buffer for 16 hours at 37°C prior to staining the gel. The starting material was found to contain both gelatinase A and B, however, no gelatinase activity was detected in the zinc-purified fibronectin by this assay (fig.6.1).

The optimum coating concentration for type I collagen to form a substantial barrier to HT1080 cell invasion towards 10% serum over 24 hours was determined by serial dilution (fig.6.2), however, cells invaded more readily through CL-3 cleaved type I collagen when coated at the same concentration as native type I collagen (fig.6.3). Zymographic analysis of the conditioned medium from these experiments revealed an increase in active gelatinase A production by cells invading through collagen coated filters compared to cells migrating through uncoated filters, but no difference in gelatinase levels or activity between cells invading through native or cleaved collagen (fig.6.4). The degree of collagen cleavage was assessed on each occasion by SDS

PAGE analysis to ensure that cells were presented with a clear difference in substrate (data not shown).

HT1080 cell invasion through type I collagen and the 3/4 and 1/4 fragments of type I collagen was assessed during 6 hour assays. The substrates were coated onto the filters on an equimolar basis to ensure a fair comparison, however, HT1080 cells invaded more readily through the cleaved fragments than through the intact collagen (fig.6.5). In order to check that any increase in cell invasion through the fragments was not due simply to a reduction in protein coating the filters, BSA was added back to the fragment solutions prior to coating the filters to bring the final protein concentration to equal that of native type I collagen. No significant difference in cell invasion through fragments alone and fragments + BSA was found (fig.6.5), so BSA was excluded from future experiments.

In order to check that the cleaved collagen fragments were adhering to the plastic filters in a similar ratio to the native collagen, native and CL-3 cleaved ^{14}C -labelled collagen was air-dried onto the filters overnight at room temperature, as described earlier in this chapter for unlabelled collagen. The residual liquid after the rehydration stages of the protocol (prior to addition of cells) was collected from both the filters coated with ^{14}C -labelled collagen, and the filters coated with the cleavage mixture of ^{14}C -labelled collagen, and separately analysed in a scintillation counter. The percentage of native or cleaved collagen adhering to the filters at the end of the rehydration stages was estimated from the number of counts released into the wash liquid during rehydration, compared to the number of counts ~~released by~~ the starting volume of ^{14}C -labelled collagen. Samples were analysed in duplicate. The total counts per minute ~~released by~~ 100 μl of ^{14}C -labelled collagen (100 $\mu\text{g}/\text{ml}$), the starting volume of collagen, was 6,630 and 7,617. The total counts released by the uncleaved ^{14}C -labelled collagen during rehydration of the collagen film was 120 and 122 counts per minute. The total counts released by the CL-3 cleaved ^{14}C -labelled collagen during rehydration of the film was 570 and 542 counts per minute. Although more counts were released by the cleavage mixture of collagen fragments compared to the uncleaved collagen, these were still less than 5% of the total available counts. Therefore, it was estimated that 95% of the collagen fragments remained stuck to the plastic filters during rehydration.

The increase in HT1080 cell invasion through type I collagen fragments compared to native type I collagen was not affected by preincubation of cells with an anti- α_2 integrin antibody (fig.6.6) or an RGD-containing peptide (fig.6.7). However, the number of cells invading through the fragments could be reduced by a small but statistically

significant amount if TIMP-2 was added to the cells at the start of the invasion assay. Interestingly, addition of TIMP-2 had no effect on the number of cells migrating through uncoated filters. Inclusion of the serine proteinase inhibitor aprotinin to the cells with TIMP-2 had no additive effect on reduction in cell invasion (fig.6.8) although it completely ablated activation of GLB secreted by cells. The reduction in HT1080 cell invasion through the collagen fragments when a synthetic MMP inhibitor, (CT1746), was added to the invasion assay was comparable to the level of cell inhibition caused by TIMP-2 (data not shown).

Gelatinized type I collagen was compared to native type I collagen as a barrier to cell invasion. It was found that a greater number of cells were able to invade through the denatured substrate but that less cells invaded through gelatin than through the cleaved fragments (fig.6.9). Cell invasion through gelatin or the collagen fragments was not inhibited by an anti- α_2 integrin antibody (fig.6.10) or an RGD-containing peptide (data not shown).

Laminin-1, used at high concentrations (100 $\mu\text{g/ml}$ or more), was also found to be a chemoattractant for HT1080 cells. In one experiment laminin-1 was used as a chemoattractant to investigate if HT1080 cells were using a fibronectin-bridge mechanism to move more readily through the collagen fragments than through intact collagen. However, many more cells invaded through the cleaved fragments towards the laminin, compared to cell invasion through the native collagen (fig.6.11). Since the ratio of cells invading through the fragments relative to uncleaved collagen in response to laminin-1 or serum was very similar, the possibility that HT1080 cells were using a fibronectin bridge to facilitate movement through the fragments was discounted.

Discussion

These data clearly show that collagenase-cleavage of type I collagen dramatically affects HT1080 cell invasion through this substrate. Cell motility towards at least two chemotactic proteins was consistently upregulated through the collagen fragments relative to cell motility through uncleaved type I collagen during the assay periods. This suggests that HT1080 cells can detect the change in extracellular environment following collagenase degradation of the substrate and modify their behaviour accordingly.

Plasma fibronectin was found to be a chemoattractant for HT1080 cells during the 24 hour invasion assays but the number of cells invading moving towards FN during invasion assays was found to be very variable. Due to the inconsistency of FN as a chemoattractant, 10% serum was used in its place for cell invasion through type I collagen. Analysis of the medium from upper wells of these experiments showed that gelatinase A activation was upregulated by cells confronted by native or cleaved type I collagen compared to cells migrating through uncoated filters. Type I collagen has been shown to upregulate GLA activation by these cells (Azzam and Thompson, 1992), although GLA is not generally considered to be a collagenolytic enzyme. Interestingly, there was no obvious difference in GLA production or activation in response to native or collagenase-cleaved collagen. Cleaved collagen can be considered as gelatin (as both fragments produced melt below 37°C), so this would be a likely target for active GLA produced by invading cells. This may have been the case during the 24 hour assays performed here as there was an increase in HT1080 cell invasion through the cleaved type I collagen.

HT1080 cells invaded significantly more readily through the cleaved fragments than through native collagen when compared on an equimolar basis, and this was not simply due to a reduction in protein coating the filters. Cell invasion through gelatin was also upregulated compared to invasion through native collagen but due to renaturation of the gelatin, the number of cells invading through gelatin was not as high as through cleaved type I collagen fragments.

Incubation of cells with 6F1 anti- α_2 integrin antibody had no effect on cell invasion through the cleaved collagen fragments. Because the level of HT1080 cell invasion through type I collagen coated at 100 $\mu\text{g/ml}$ was so low it was not possible to detect whether the antibody or peptide had any effect on cell invasion, although since HT1080 cells use $\alpha_2\beta_1$ integrin to attach firmly to native collagen, reduction in cell attachment by 6F1 may be speculated to have led to an increase in cell motility.

It is well known that fibronectin binds to denatured fibrillar collagens with higher affinity than it does to the native proteins (reviewed by Kühn, 1987), and cells have been found to interact with denatured collagen via a fibronectin-bridge (Tuckwell et al., 1994). Since HT1080 cells express $\alpha_5\beta_1$ integrin, which is a RGD-dependent receptor for fibronectin, the possibility that cells were using such a mechanism to facilitate cell motility through the cleaved type I collagen fragments was tested. However, inclusion of an RGD-containing peptide into the serum-containing assays had no effect on cell motility through the fragments, and neither did use of laminin-1 as a chemoattractant. These results suggest that HT1080 cells probably do not use fibronectin as a mechanism to interact with collagenase-generated type I collagen fragments, particularly since cell interactions with laminin-1 are likely to be via an RGD-independent receptor (such as $\alpha_6\beta_1$ integrin).

Addition of TIMP-2 to the invasion assay caused a small but significant decrease in HT1080 cell invasion through the collagen fragments. This reduction of motility was recorded using a relatively high dose of TIMP-2 (2 μM), although this concentration was still within physiological range. Clearly, TIMP-2 did have minor part to play in reducing cell motility through the cleaved collagen fragments, possibly by inhibition of endogenous MMPs produced by HT1080 cells. Inhibition of serine proteinases also had no effect on cell motility, but did completely inhibit activation of GLB (data not shown). This data lend support to the hypothesis that active MMPs may facilitate cell

invasion, but these are not the only factors involved in mediating cell motility through cleaved type I collagen.

Table 1

Chemoattractant	Mean Number Cells Migrated in 24h +/- Standard Deviation
CM	95,000 +/- 34,559
CM*	10,000 +/- 8,049
CM* + FN	181,000 +/- 22,053
FN + insulin	42,000 +/- 14,292
GLA + GLB + insulin	795 +/- 524
FN*	27,000 +/- 10,894

Key: CM = 3T3 cell conditioned medium; CM* = conditioned medium after purification over gelatin-agarose; FN = plasma fibronectin (20 µg/ml); insulin = 5.0 µg/ml; GLA and GLB = 1.0 µg/ml; FN* = fibronectin after purification over zinc-chelated Sepharose.

NIH 3T3 cell conditioned medium (CM) was an efficient chemoattractant for HT1080 fibrosarcoma cells, however, the CM contained gelatinases. These were removed by batch affinity chromatography using a gelatin-agarose matrix. However, this had the effect of dramatically reducing HT1080 cell migration towards the CM. Fibronectin is also binds to gelatin-agarose, therefore, purified human plasma fibronectin was added back to the CM to try to restore the chemotactic ability of the CM. This proved to be a success, so the chemotactic ability of fibronectin was tested with insulin in DMEM. A significant number of HT1080 cells migrated towards the fibronectin and insulin solution, however, it was noted that the purified fibronectin also contained gelatinases. The possibility that the gelatinases could be chemoattractants was tested, however, these proteins did not stimulate many cells to migrate during the assay period. Gelatinases were removed from fibronectin using a zinc-chelated Sepharose column. The ultra-pure fibronectin was tested for chemotactic ability and found to retain most of its chemotactic properties for HT1080 cells.

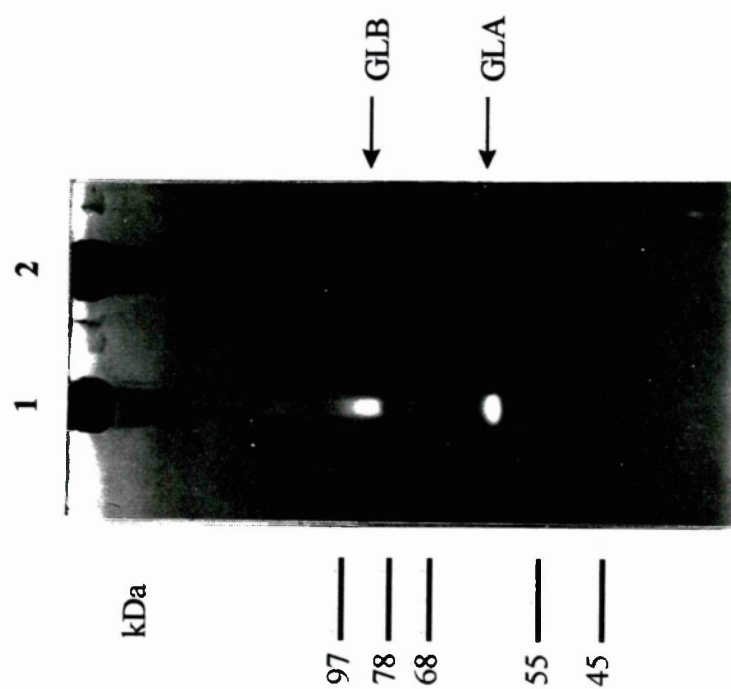


Fig. 6.1: Gelatin zymogram of purified fibronectin prior to further purification over a zinc-chelated column (lane 1). This method removed all traces of gelatinase A and B (lane 2).

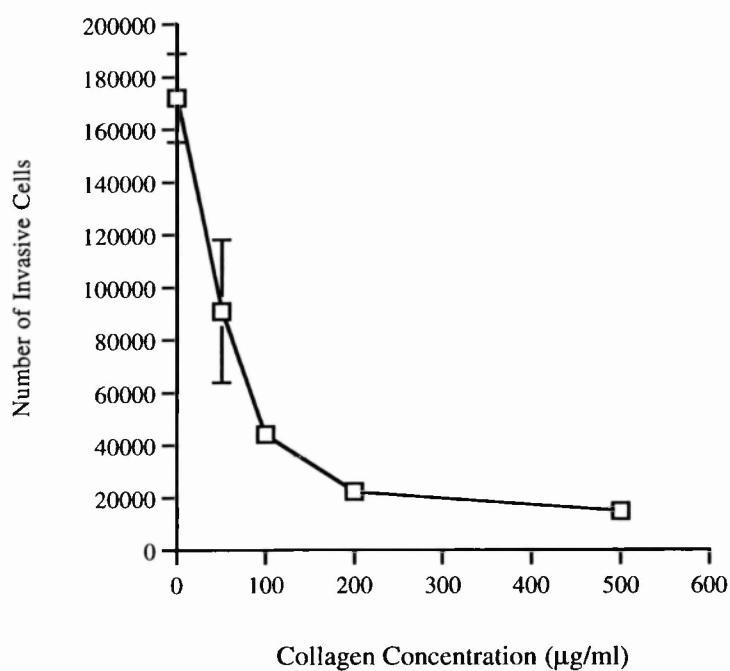


Fig.6.2: HT1080 cell invasion through type I collagen after 24 hour assay decreased with increasing type I collagen coating concentration. Where error bars are not shown, bars are smaller than the point markers.

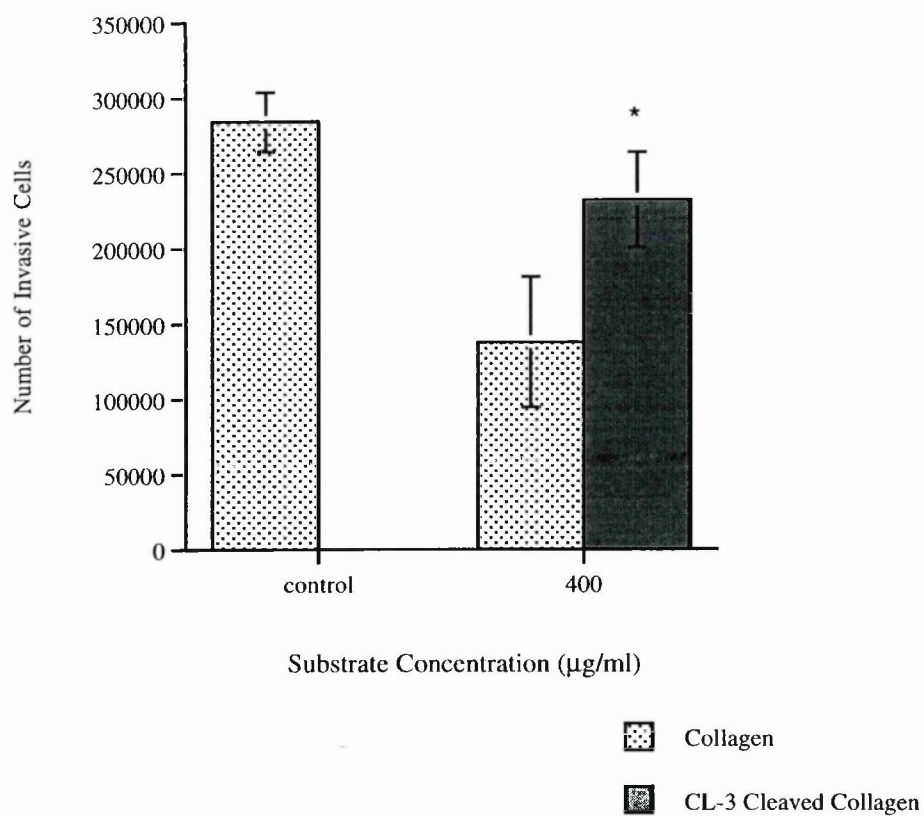


Fig.6.3 : More HT1080 cells invaded after 24 hour assay through collagenase-3 cleaved type I collagen than through native collagen (* $p < 0.05$). Migration through uncoated filters is denoted as "control". Values shown are the mean of triplicates \pm standard deviation.

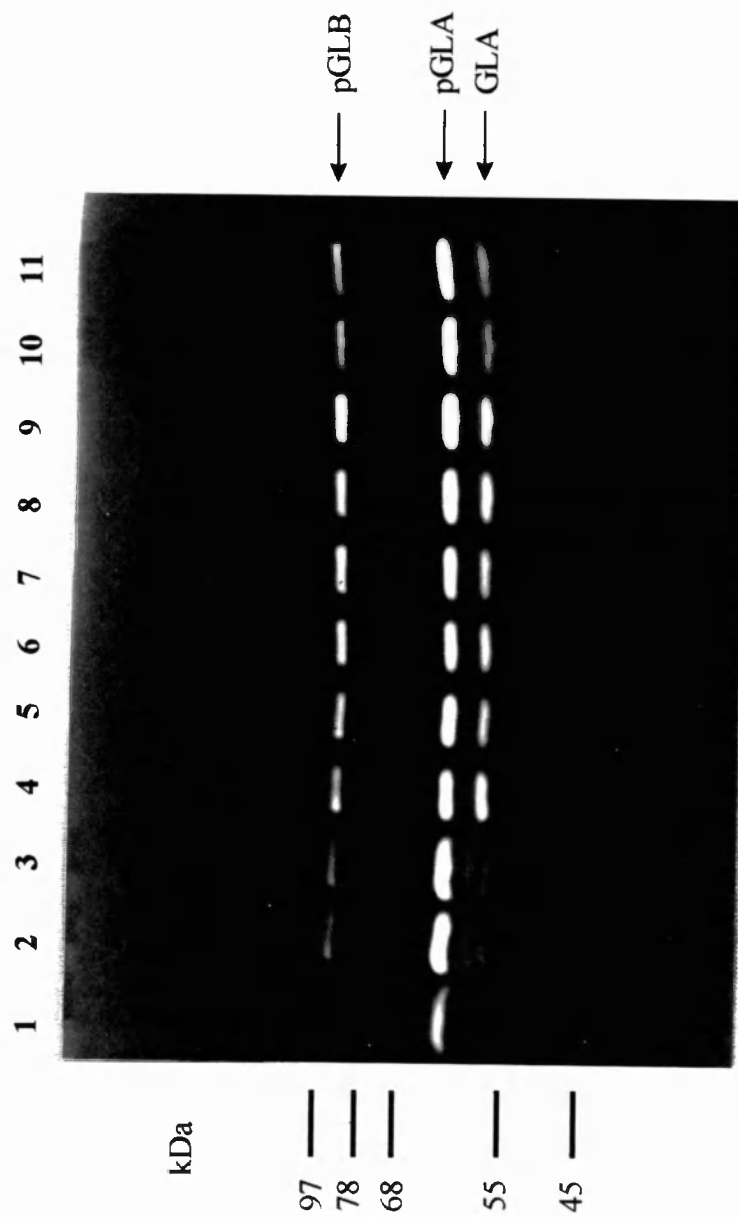


Fig. 6.4: Gelatin zymogram of conditioned medium from cells migrating towards 10% FCS through uncoated filters (lanes 2 and 3), and invading through native collagen (lanes 4 and 5) or the purified 3/4 fragment (lanes 6-8) and the 1/4 fragment (lanes 9-11). Lane 1 contains a sample of medium taken from the upper chamber of a well at the end of the 24 hour assay which had no cells in it. Cells migrating through uncoated filters produced GLA which was primarily in the pro-form, in contrast to cells invading through a collagen or collagen fragment barrier, which converted some GLA to the active form.

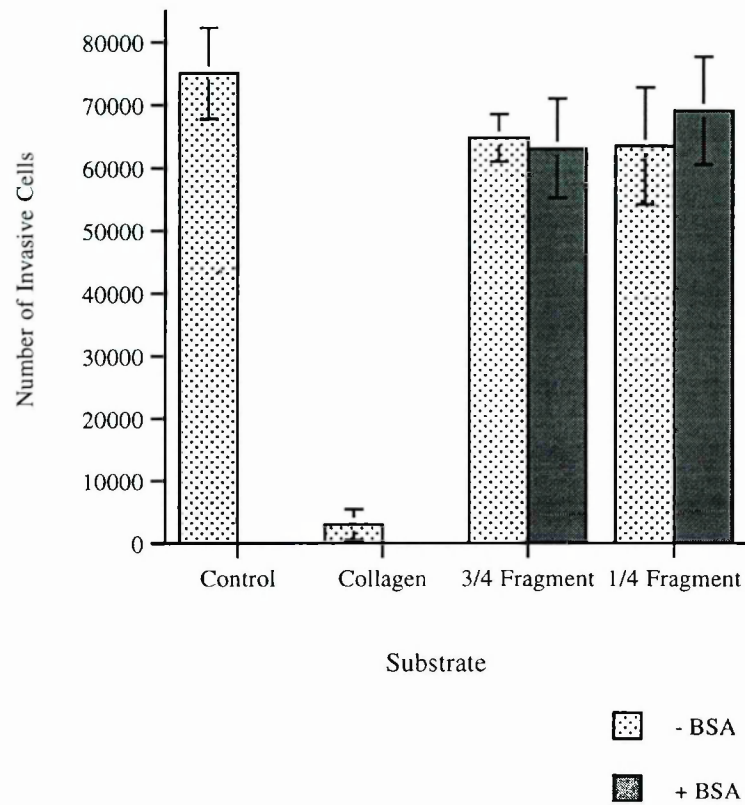


Fig.6.5 : Addition of BSA to the 3/4 and 1/4 fragments to increase the coating concentration to equal that of native collagen (100 $\mu\text{g/ml}$) had no effect on HT1080 cell invasion after 6 hours. Cell migration through uncoated filters is denoted by "control". Values shown are the mean of triplicates, +/- standard deviation.

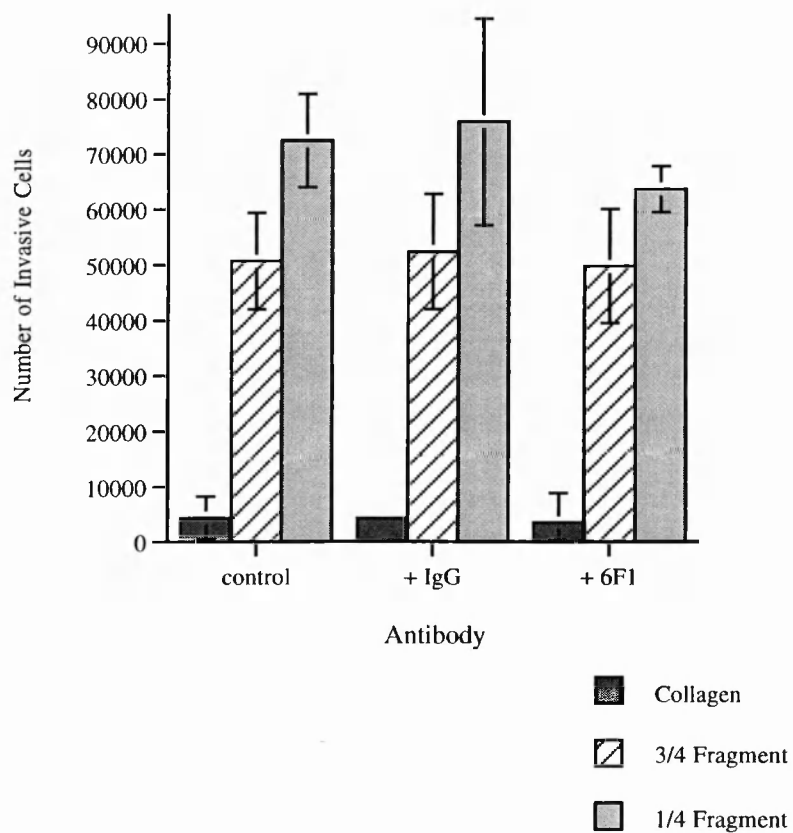


Fig.6.6 : Addition of an anti- α_2 integrin antibody (6F1; 10.0 $\mu\text{g/ml}$) had no effect on HT1080 cell invasion through native type I collagen and collagen fragments over 6 hours. Cell invasion in the absence of antibodies is denoted by "control". Values are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are too small to visualize on this scale.

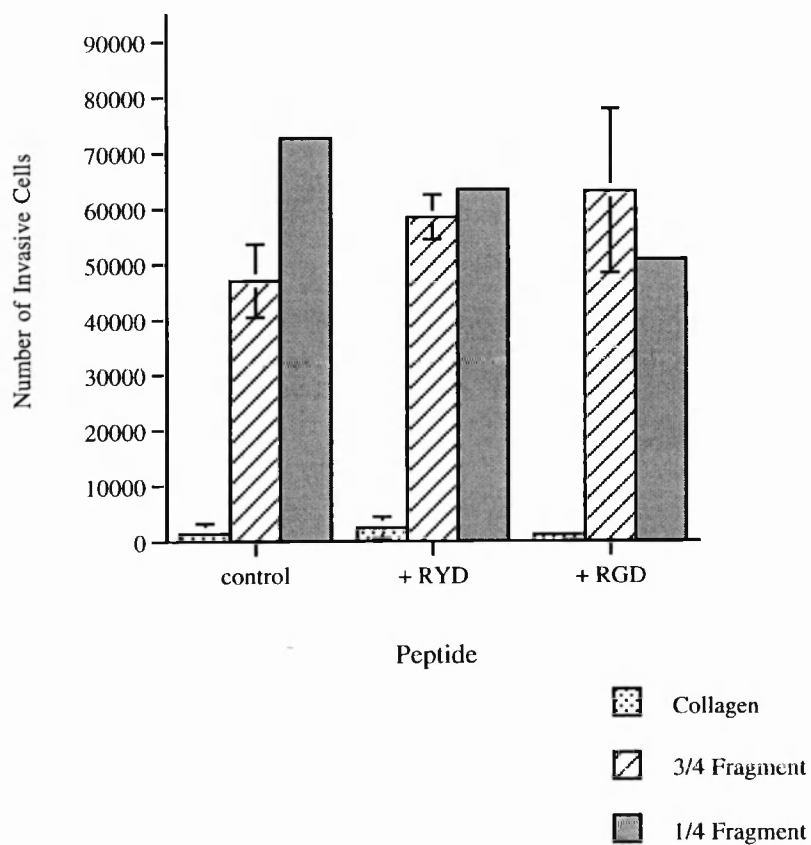


Fig.6.7 : Addition of an RGD-containing peptide or an RYD peptide control (20.0 $\mu\text{g/ml}$) had no effect on HT1080 cell invasion through native type I collagen and collagen fragments over 6 hours. Cell invasion in the absence of peptide is denoted as "control". Values are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are too small to visualize on this scale.

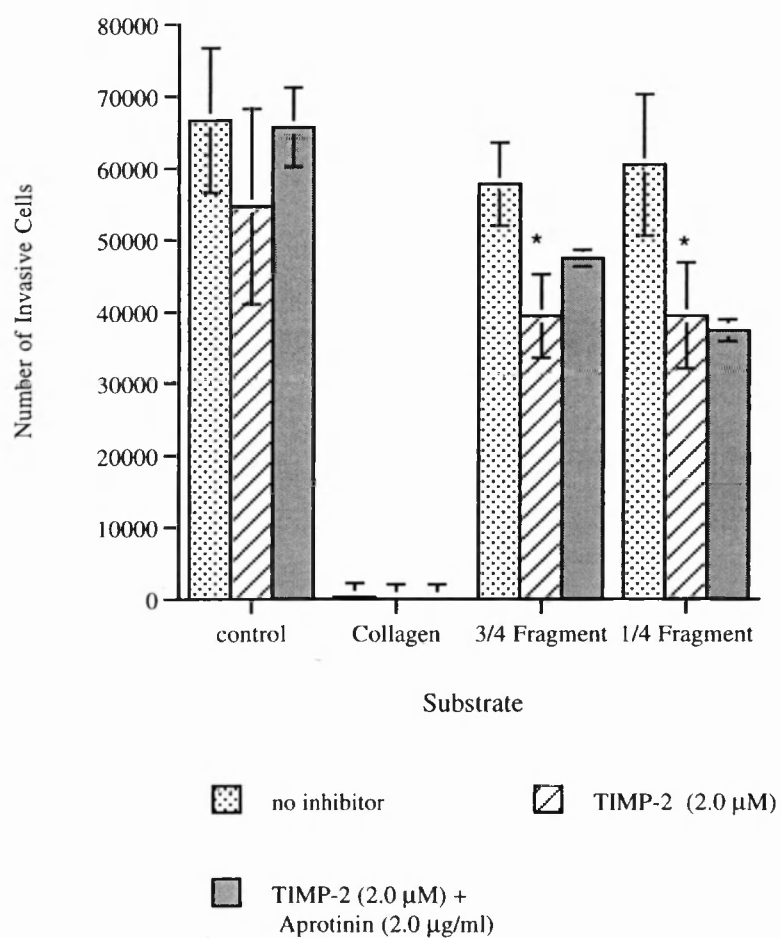


Fig.6.8 : HT1080 invasion over 6 hours was significantly inhibited by addition of TIMP-2 (* $p<0.05$). Inclusion of aprotinin had no additive effect on the decrease in cell invasion. Values shown are the mean of triplicates, \pm standard deviation.

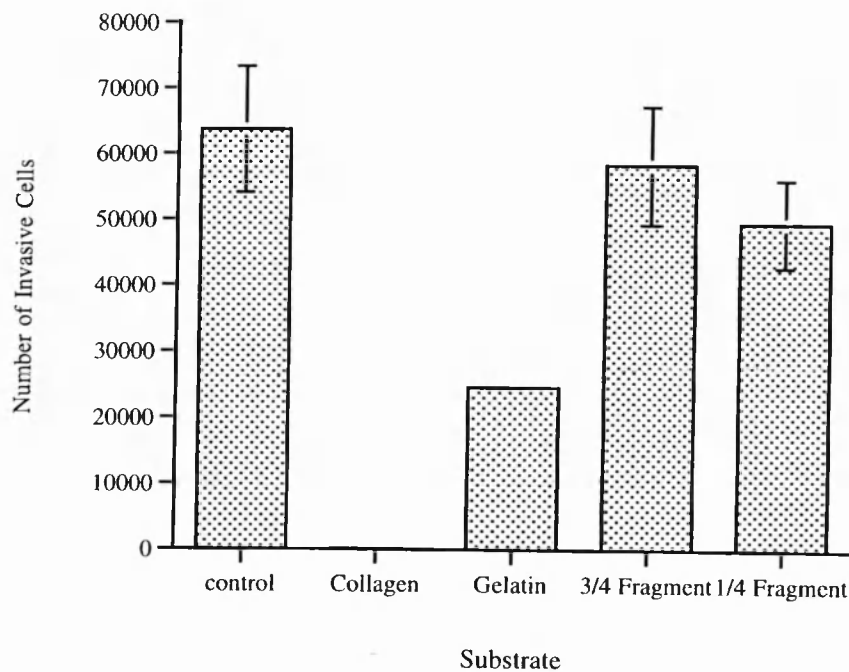


Fig.6.9 : Fewer HT1080 cells were able to invade through gelatin over 6 hours than through type I collagen fragments. Migration of cells through uncoated filters is denoted by "control". Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are too small to be visualized on this scale.

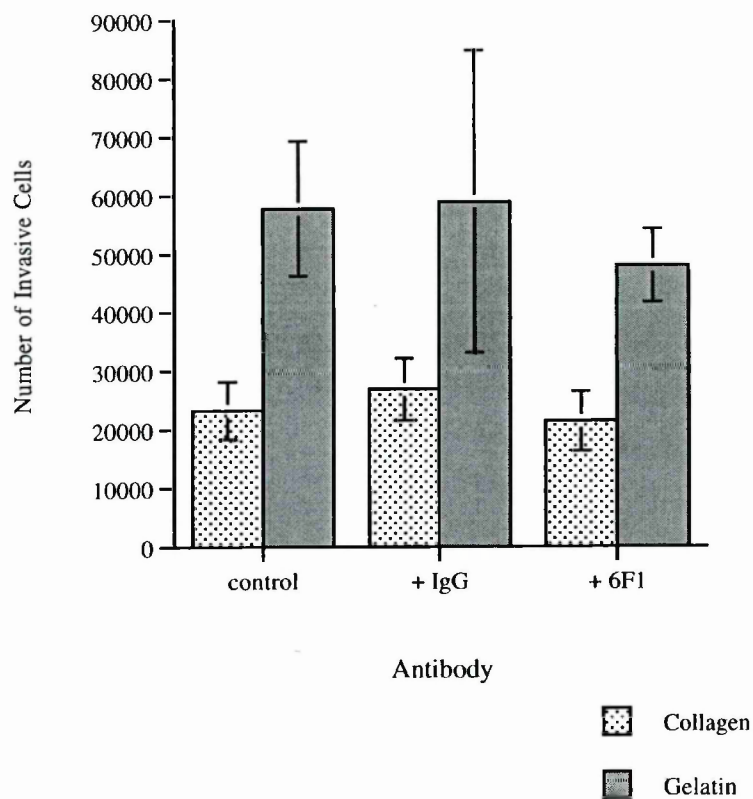


Fig.6.10 : HT1080 cell invasion through type I collagen and gelatin over 6 hours was not affected by addition of an anti- α_2 integrin antibody (10.0 $\mu\text{g/ml}$). More cells invaded through both collagen and gelatin in this assay compared to Fig.6.9, however, the proportion of cells invading through the native and denatured substrate remained the same. Invasion of cells in the absence of antibody is denoted by "control". Values shown are the mean of triplicates, \pm standard deviation.

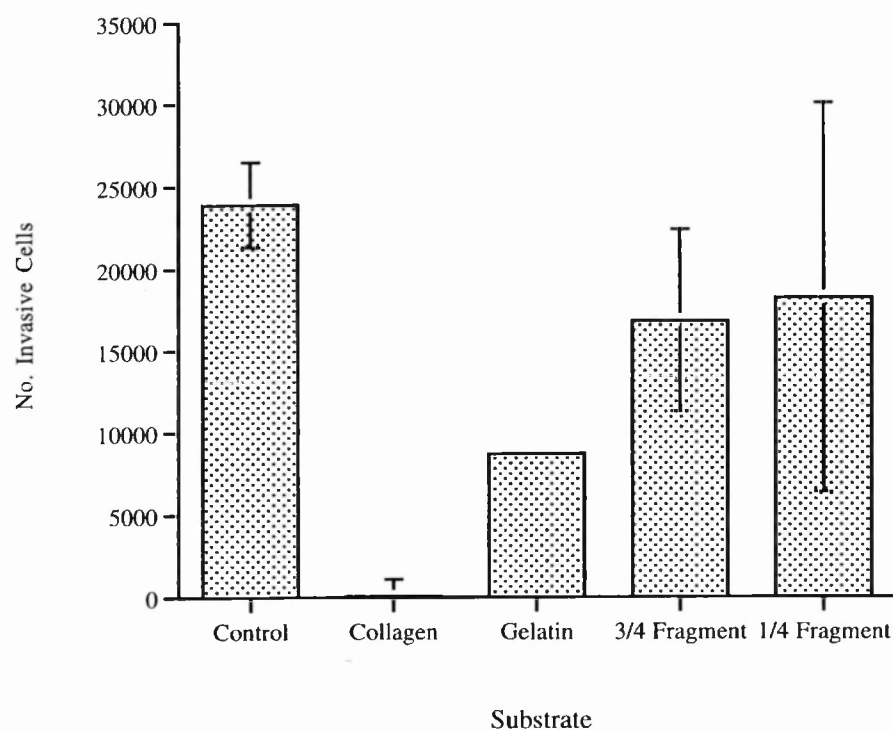


Fig.6.11 : Laminin-1 was chemotactic for HT1080 cells invading through gelatin and the collagen fragments. Cell invasion through gelatin and the fragments was not downregulated compared to invasion through native collagen, suggesting that cells probably do not use a fibronectin-bridge mechanism to invade through the fragments towards serum. Migration of cells through uncoated filters is denoted by "control". Values shown are the mean of triplicates, +/- standard deviation.

Chapter 7

Integrins as Novel Substrates for MMP degradation

Introduction

Several groups have reported increased human platelet (Pidard et al., 1991; Si-Tahar et al., 1997) and human melanoma cell (Fujii and Imamura, 1995) adhesion to ECM molecules after incubation of the cells with serine proteinases such as trypsin or plasmin, possibly due to proteolytic activation of integrin or exposure of sequestered integrin.

In contrast, Ray and Stetler-Stevenson (1995) reported that preincubation of A2058 human melanoma cells with active gelatinase A resulted in decreased cell attachment to fibronectin and vitronectin. Addition of recombinant human TIMP allowed increased binding of these cells to both substrates. The authors interpreted their results to mean that gelatinase A can directly modulate cell attachment and spreading. It was of great interest to further investigate this hypothesis using HT1080 human fibrosarcoma cells, A375M human melanoma cells and A2058 human melanoma cells. Cells were incubated with various MMPs prior to attachment to type I collagen and fibronectin. Attachment assays were performed essentially as described in Chapter 4.

The direct degradative effect of active MMPs on integrins was also tested in a controlled system. Purified integrins were incubated with active MMPs and serine proteinases at known enzyme-substrate ratios, since proteolytic modification of integrins could account for the increased cell adhesion described by other authors.

Methods

Cell Lines and Tissue Culture

A2058 human melanoma cells were purchased from the American Tissue Culture Collection. Human HT1080 fibrosarcoma cells and A375M melanoma cells were obtained as described in Chapter 2. Cells were maintained in DMEM supplemented with 10% foetal calf serum as described in Chapter 2. Cells were passaged 24-48 hours prior to assay and harvested at approximately 80% confluency. Cells were suspended in serum-free DMEM and centrifuged at 1500 g for 5-10 minutes to remove the trypsin:EDTA solution. The cell pellet was resuspended in serum free DMEM, cells counted using a haemocytometer and diluted in DMEM to the desired concentration.

Purified Integrins

Human platelet $\alpha_2\beta_1$ integrin was purified as described in Chapter 3. Chimeric $\alpha_7\beta_1$ integrin was isolated from human 293 kidney cells producing β_1 integrin and transfected with mouse α_7 integrin (Echtermeyer et al., 1996), and was a kind gift of Dr. Helga Von der Mark, Institute of Experimental Medicine, Friedrich-Alexander University, Erlangen-Nuremberg, Germany. Human $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins were isolated from human placenta, and were kind gifts of Dr. David Simmons, University of Oxford, Oxford, UK and Dr. Simon Goodman, Merck Laboratories Ltd, Darmstadt, respectively.

MMP Activation

MMPs were activated essentially as described by Murphy et al. (1991), with minor modifications. Briefly, pGLA was activated by incubation with 2 mM amino phenyl mercuric acetate (APMA) for 1 hour at 25°C, pGLB was activated by incubation with 1 mM APMA for 1 hour at 37°C. The cytotoxic APMA was removed from the gelatinase

solution by size-separation using Sephadex (Sigma) spin columns. Activated GLA and GLB could be stored at -20°C for up to one week without losing proteolytic activity as determined by gelatinase assay as described in Chapter 2.

pSL-1 was activated by incubation with trypsin (5.0 µg/ml) for 30 minutes at 25°C, the reaction was quenched by addition of 50 µg/ml soyabean trypsin inhibitor (SBTI) and incubation at 4°C for 30 minutes. pCL-1 was activated by incubation with trypsin (5.0 µg/ml) and pSL-1 (0.2 µg/ml) for 30 minutes at 25°C. The reaction was quenched as for SL-1 activation. Activated SL-1 and CL-1 could also be stored at -20°C for up to one week, as determined by stromelysin assay and collagen diffuse fibril assay.

The specific activities of the active enzymes were determined by radio-labelled substrate assay in conjunction with each cell attachment assay. Collagenase diffuse fibril assay was performed as described by Cawston and Barrett (1979), Gelatinase and Stromelysin assays were performed as described by Murphy et al. (1981b), see Chapter 2. The specific activities of each enzyme upon each activation was calculated and is expressed in units/mg (one unit of each enzyme degrades one microgram of substrate in one minute at 37°C).

Cell Attachment

The methods of Ray and Stetler-Stevenson (1995) were followed as closely as possible, however their exact methodology was not clear from the paper. Therefore, attachment assays were carried out as described in Chapter 4, with some modifications. After trypsin/EDTA treatment, cells were pelleted in serum-free DMEM and resuspended in DMEM. Cells were allowed to recover from trypsin treatment for 30 minutes at 37°C in serum-free DMEM prior to MMP treatment. Recovered cells were pelleted and resuspended in DMEM containing activated MMPs (250-500 ng/ml) and incubated at 37°C for 30 minutes. Cells (6×10^5 /well) were plated onto BSA-blocked

substrates and incubated for 35 minutes at 37°C. Unattached cells were washed off and attached cells fixed and stained as described in Chapter 5. Other modifications included addition of BSA to the cell-MMP incubation and longer assay periods. MMPs were generally included in the attachment assay as removal by centrifugation resulted in uneven loss of cells from each MMP incubation, rendering comparison of the final data difficult.

MMP integrin-degradation Assays

Recombinant human trypsin, plasmin and plasminogen were purchased from Sigma, USA. Plasminogen was activated by incubation with streptokinase (Kabi Ltd, UK) at 100:1 plasminogen:streptokinase ratio at 37°C for 15 minutes. Gelatinase A and B, stromelysin-1 and collagenase-1 were activated as described earlier in this chapter. Collagenase-3 was activated by incubation with 1 mM APMA for 30 minutes at 37°C (Knäuper et al., 1996a). MMP activity was assessed in parallel to the integrin assays by ¹⁴C-labelled substrate assay as described in Chapter 2. Plasmin activity was assessed using the ¹⁴C-labelled casein assay also used for detection of stromelysin activity.

Activated MMPs in dilution buffer (10 mM Tris pH7.5, 10 mM CaCl₂, 0.05% Brij, 0.02% sodium azide) were added to integrins in TBS, 1 mM MgCl₂, 1 mM MnCl₂ (0.5 µg integrin/tube, 100:1 and 10:1 integrin:MMP molar ratio) and incubated for 16 hours at 37°C. The cleavage mixtures were analysed by 7% SDS PAGE followed by silver staining.

Results

In the absence of MMPs, HT1080 cells attach to native type I collagen under the assay conditions described above. Therefore these were the initial cell line and substrate of choice to test the hypothesis. Cells were incubated with active GLA, GLB, SL-1 or CL-

1 at 500 µg/ml (fig.7.1) or 250 µg/ml (data not shown), however, no reduction in HT1080 cell attachment to type I collagen was observed.

A human melanoma cell line was utilised by Ray and Stetler-Stevenson (1995), so the effects of MMP incubation on A375M cells was tested next. After establishing that more of these cells adhered to fibronectin than to laminin-1 under the assay conditions (data not shown), A375M cell attachment to fibronectin after incubation with MMPs (500 ng/ml) was examined. A small decrease in cell attachment after MMP-incubation was recorded in one out of two experiments (fig.7.2). MMP-incubation did not have any effect on cell attachment on the other occasion (data not shown). Although a small decrease in cell adhesion (approximately 10%) had been observed on one occasion, this was not as significant as the dramatic reduction in cell adhesion recorded by Ray and Stetler-Stevenson (approximately 80%). Therefore, it was decided to make use of the same cell line that these researchers had used and introduce some subtle variations in the assay procedure in an attempt to mimic these researchers methods.

A2058 human melanoma cells adhere almost as well to laminin-1 as to fibronectin under the above assay conditions in the absence of MMPs (data not shown), however, as Ray and Stetler-Stevenson had recorded a decrease in adhesion to fibronectin it was decided to use only fibronectin. Four out of five experiments recorded no effects on cell attachment to fibronectin after incubation with MMPs, although addition of BSA (1.0 mg/ml) into the cell-MMP incubation slightly increased the overall levels of cell attachment (fig.7.3). A single experiment showed a small reduction (approximately 10%) in cell attachment following MMP-incubation, however the result of the positive control for this experiment was much lower than anticipated and therefore this experiment was discounted (data not shown).

Human platelet $\alpha_2\beta_1$ integrin was incubated with active GLA, GLB, SL-1 and CL-1 at 10:1 and 100:1 integrin:MMP molar ratio and the cleavage mixtures analysed on 7%

polyacrylamide gels (fig.7.4); specific activities (SA) of the MMPs: GLA=8615, GLB=10813, SL-1=342, CL-1=699 units/mg). No obvious $\alpha_2\beta_1$ integrin degradation was detected as the result of several assays. Further experiments examined the effects of CL-3, MT1-MMP and MT2-MMP on this integrin (fig.7.5 and 7.6). Again, hardly any integrin degradation was visible by protein gel analysis. The proteolytic effects of the serine proteinases plasmin, plasminogen and trypsin on $\alpha_2\beta_1$ integrin were also tested (fig.7.7); SA plasmin=120 units/mg). Trypsin was found to efficiently degrade $\alpha_2\beta_1$ integrin at both 10:1 and 100:1, integrin:proteinase ratio, however, neither plasmin or plasminogen had any detectable effect on $\alpha_2\beta_1$ integrin structure.

Human $\alpha_v\beta_3$ integrin was incubated with active GLA, GLB, SL-1, CL-1 and CL-3 and analysed on 7% polyacrylamide gels (fig.7.8; SA GLA=13026, GLB=8144, SL-1=117, CL-1=192 and CL-3=1694 units/mg). None of these proteinases appeared to degrade $\alpha_v\beta_3$ integrin. Human $\alpha_7\beta_1$ integrin was also included in this experiment but no MMP degradation was recorded (data not shown). Finally, human $\alpha_v\beta_5$ integrin was incubated with GLA and GLB and analysed on a 5-15% polyacrylamide gradient gel (fig.7.9). This integrin was also found to be resistant to gelatinase degradation.

Discussion

Ray and Stetler-Stevenson (1995) reported that incubation of A2058 melanoma cells with activated GLA resulted in a dramatic decrease in cell adhesion to fibronectin. The aim of this study was to determine if their results were due to MMP degradation of one type of cell-surface receptors for ECM components, the integrins. Initially, the effect of cell incubation with various active MMPs, including gelatinase A, on cell attachment to ECM ligands was assessed. However, the ability of three different human cell lines, including A2058 cells, to attach to type I collagen or fibronectin did not appear to be inhibited by pre-incubation of cells with activated MMPs. This may be due to

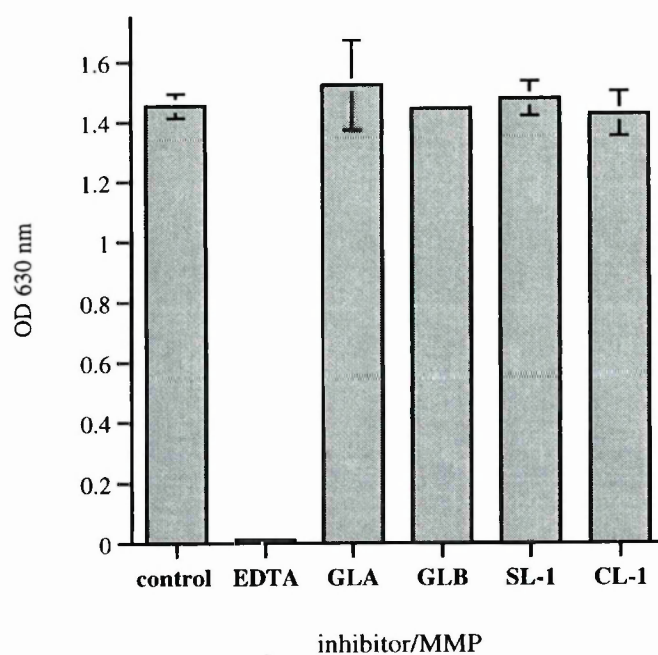
differences in the methodology used in these experiments, as the exact method used by the above authors to produce their results is not clearly documented in their paper.

MMPs degradation of ECM proteins is well documented (reviewed by Murphy and Reynolds, 1993). These enzymes may have a direct role to play in modulation of cell adhesion and motility during processes such as tumour progression, at the level of substrate cleavage and subsequent disruption of cell-substrate interactions (Mignatti and Rifkin, 1993; Crawford and Matrisian, 1994; Stetler-Stevenson, 1994; Heino, 1996). However, it would not be inconceivable for MMPs to have more subtle roles, for example by cleavage of cell-surface receptors for ECM proteins, which could also lead to reduction in cell adhesion. Reinartz et al. (1995) reported that incubation of a human keratinocyte cell line with plasmin abrogated $\alpha_v\beta_5$ integrin-mediated cell attachment to vitronectin. However this group could find no evidence that plasmin had a direct effect on the integrin receptors and concluded that degradation of the ECM substrate caused the reduction in cell attachment. Therefore, it cannot be ruled out that the reduction in cell attachment could be due to proteolytic degradation of other cell-surface proteins, such as syndecans.

The possibility that integrins might be novel substrates for MMPs and/or serine proteinases was tested directly by incubation of isolated integrins with excess active enzymes. It would have been preferable to test the effects of MMPs on the classical fibronectin receptors (such as $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins) in order to relate the isolated protein assays with the data from the cell attachment to FN assays. However, these integrins were not readily available and therefore the effect of MMPs on another FN receptor, $\alpha_v\beta_3$ integrin, was tested along with $\alpha_2\beta_1$, $\alpha_7\beta_1$ and $\alpha_v\beta_5$ integrins. Trypsin was able to efficiently cleave $\alpha_2\beta_1$ integrin under the assay conditions described above, however, another serine proteinase, plasmin, did not show any degradative ability towards this integrin. None of the MMPs tested appeared to have any proteolytic effect on human $\alpha_2\beta_1$, $\alpha_7\beta_1$, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins in solution. This was an interesting

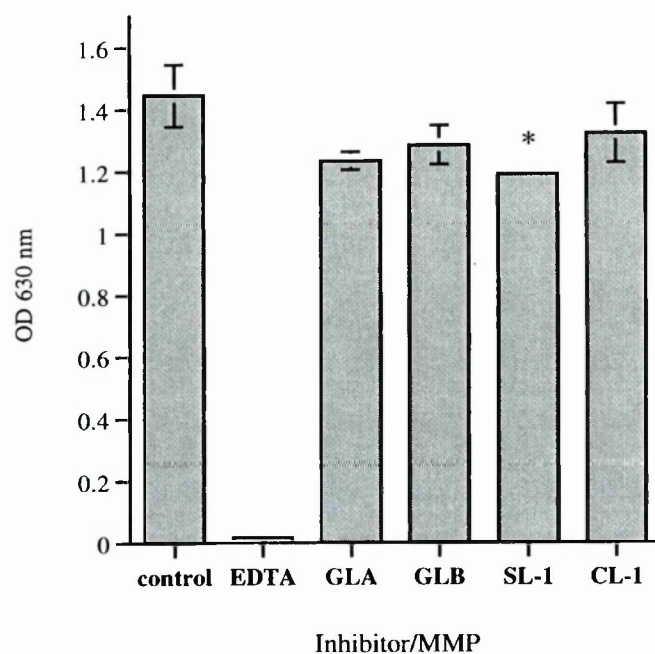
observation, as the transmembrane and cytoplasmic domains of these integrins that would not normally be exposed to active MMPs in the extracellular environment, were available to the MMPs in the test-tube but were not degraded. However, a recent report by von Bredow et al. (1997) showed that matrilysin can degrade human β_4 integrin subunit transfected into human prostate carcinoma cells, both in cell lysates and on the surface of cells grown on glass coverslips. This same study showed that receptor-degrading activity of matrilysin was specific for the β_4 integrin, as the β_1 integrin subunit was resistant to degradation by this MMP. The authors note that the sequence of the β_4 integrin subunit (as listed in the EMBL protein database) contains two candidate matrilysin cleavage sites, but the β_1 subunit does not contain any appropriate sequences at which matrilysin could cleave.

The inability of MMPs to degrade the integrin class of cell-surface ECM receptors is likely to be of great significance to cell-matrix interactions in areas of tissue undergoing active remodelling, particularly in relation to their function as signal-transducing proteins able to relay information about a cell's extracellular environment. Integrin resistance to proteinase degradation during ECM remodeling may facilitate cell detection of, and reaction to, changes in pericellular conditions (Auer and Jacobson, 1995; Clark and Brugge, 1995; Richardson and Parsons, 1995; Dedhar and Hannigan, 1996). Loss of these information channels could lead to inappropriate cell behaviour in an altered cellular environment, potentially leading to catastrophic events such as apoptotic cell death or even gain of invasive capacity.



EDTA = 10 mM
 GLA, GLB, SL-1 and CL-1 = 500 ng/ml
 Specific Activities: GLA = 8275 units/mg
 GLB = 8834 units/mg
 SL-1 = 414 units/mg
 CL-1 = 1238 units/mg

Fig.7.1 : preincubation of HT1080 cells (6×10^5 cells/well) with active MMPs had no effect on cell attachment to type I collagen, but preincubation with 10 mM EDTA completely inhibited cell attachment. Data denoted as "control" refers to cell attachment in the absence of MMPs. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are too small to visualize on this scale.



EDTA = 10 mM

GLA, GLB, SL-1, CL-1 = 500 ng/ml

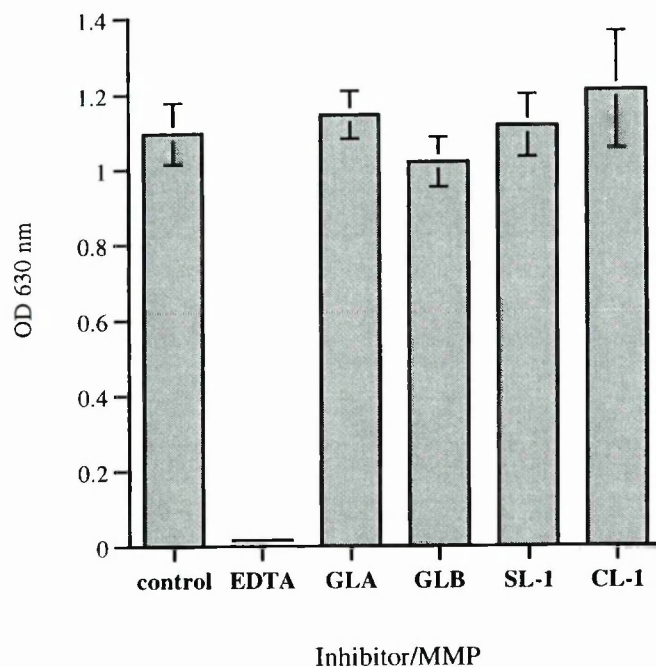
Specific Activities: GLA = 9389 units/mg

GLB = 10587 units/mg

SL-1 = 368 units/mg

CL-1 = 1000 units/mg

Fig.7.2 : Preincubation of A375M cells (6×10^5 cells/ml) with active SL-1 had a small but significant effect on cell attachment to fibronectin (* $p < 0.05$). No effect was observed after incubation with the other MMPs tested. However, incubation of cells with 10 mM EDTA completely inhibited cell attachment. Data denoted as "control" refers to cell attachment in the absence of MMPs. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are too small to visualize on this scale.



EDTA = 10 mM

GLA, GLB, SL-1, CL-1 = 500 ng/ml

Specific Activities: GLA = 9601 units/mg

GLB = 7205 units/mg

SL-1 = 362 units/mg

CL-1 = 1029 units/mg

Fig.7.3 : Preincubation of A2058 cells (6×10^5 cells/ml) with active MMPs had no effect on cell attachment to fibronectin in the presence of 1 mg/ml BSA, but incubation with 10 mM EDTA completely inhibited cell attachment. Data denoted as "control" refers to cell attachment in the absence of MMPs. Values shown are the mean of triplicates, \pm standard deviation. Where error bars are not shown, bars are too small to visualize on this scale.

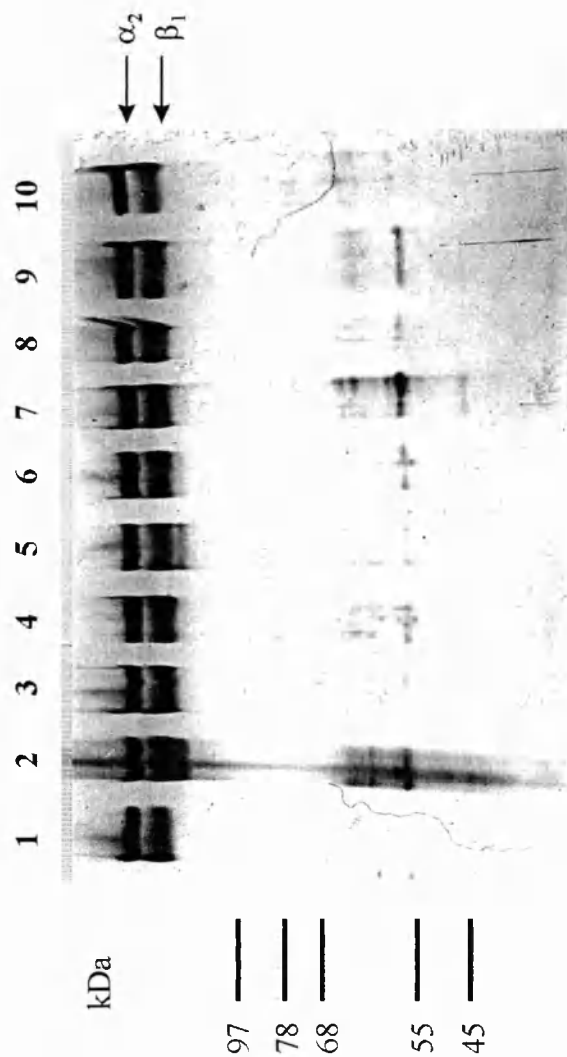


Fig.7.4: Incubation of $\alpha_2\beta_1$ integrin with MMPs for 16 hours at 37°C. Lane 1: unincubated $\alpha_2\beta_1$ integrin; 2: incubated $\alpha_2\beta_1$ integrin; 3: $\alpha_2\beta_1$ integrin + GLA (10:1); 4: $\alpha_2\beta_1$ integrin + GLA (100:1); 5: $\alpha_2\beta_1$ integrin + GLB (10:1); 6: $\alpha_2\beta_1$ integrin + GLB (100:1); 7: $\alpha_2\beta_1$ integrin + SL-1 (10:1); 8: $\alpha_2\beta_1$ integrin + SL-1 (100:1); 9: $\alpha_2\beta_1$ integrin + CL-1 (10:1); 10: $\alpha_2\beta_1$ integrin + CL-1 (100:1). None of these enzymes appeared to cleave $\alpha_2\beta_1$ integrin.

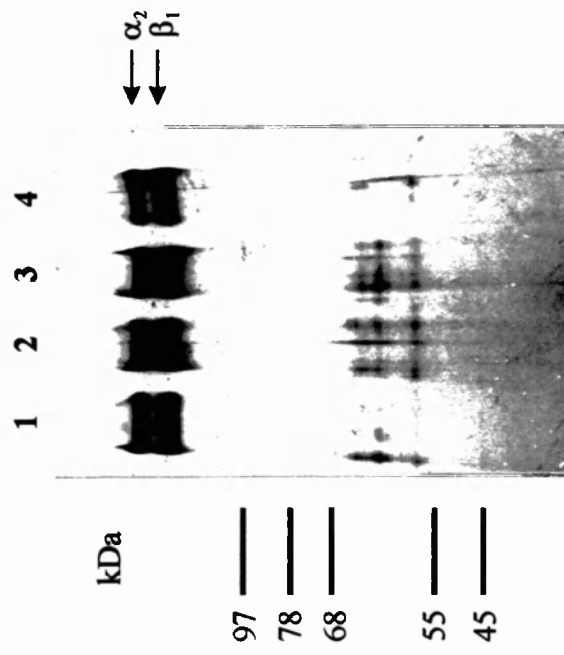


Fig. 7.5: Incubation of $\alpha_2\beta_1$ integrin with collagenase-3 for 16 hours at 37°C. Lane 1: unincubated $\alpha_2\beta_1$ integrin; 2: incubated $\alpha_2\beta_1$ integrin; 3: $\alpha_2\beta_1$ integrin + CL-3 (10:1); 4: $\alpha_2\beta_1$ integrin + CL-3 (100:1). CL-3 did not cleave $\alpha_2\beta_1$ integrin.

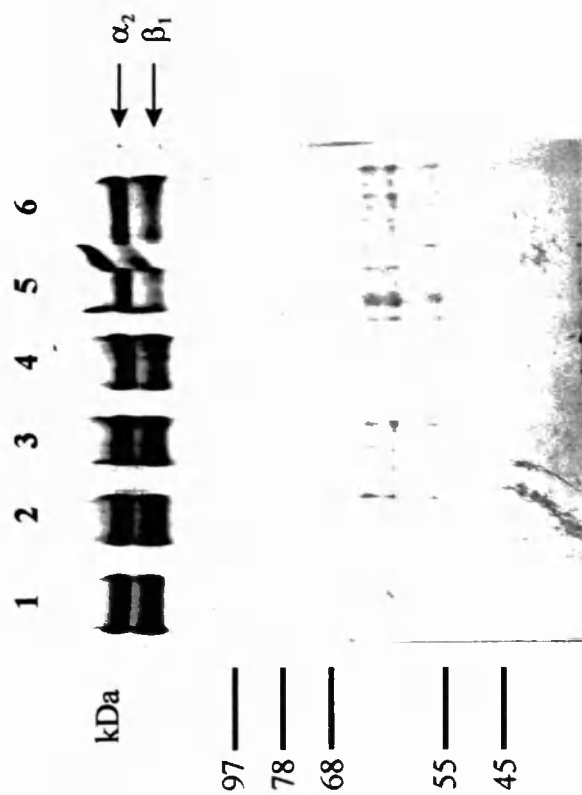


Fig. 7.6: Incubation of $\alpha_2\beta_1$ integrin with MT-MMPs for 16 hours at 37°C. Lane 1: unincubated $\alpha_2\beta_1$ integrin; 2: incubated $\alpha_2\beta_1$ integrin; 3: $\alpha_2\beta_1$ integrin + MT-1 MMP (10:1); 4: $\alpha_2\beta_1$ integrin + MT-1 MMP (100:1); 5: $\alpha_2\beta_1$ integrin + MT-2 MMP (10:1); 6: $\alpha_2\beta_1$ integrin + MT-2 MMP (100:1). Neither MT MMP appeared to cleave $\alpha_2\beta_1$ integrin.

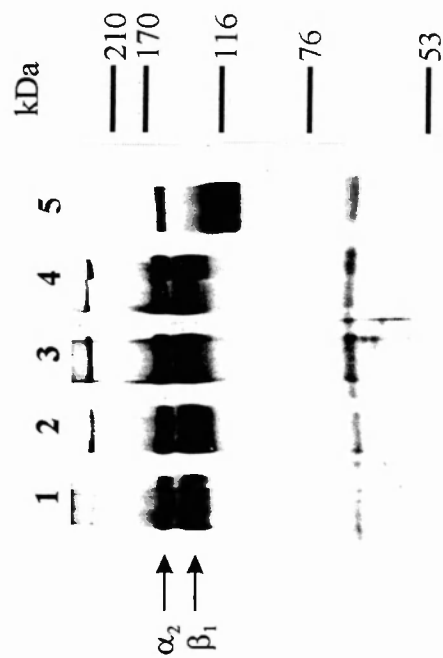


Fig. 7.7: Incubation of $\alpha_2\beta_1$ integrin with serine proteinases for 16 hours at 37°C. Lane 1: unincubated $\alpha_2\beta_1$ integrin; 2: incubated $\alpha_2\beta_1$ integrin; 3: $\alpha_2\beta_1$ integrin + plasminogen (10:1); 4: $\alpha_2\beta_1$ integrin + plasmin (10:1); 5: $\alpha_2\beta_1$ integrin + trypsin (10:1). Trypsin was able to degrade $\alpha_2\beta_1$ integrin although plasmin was not.

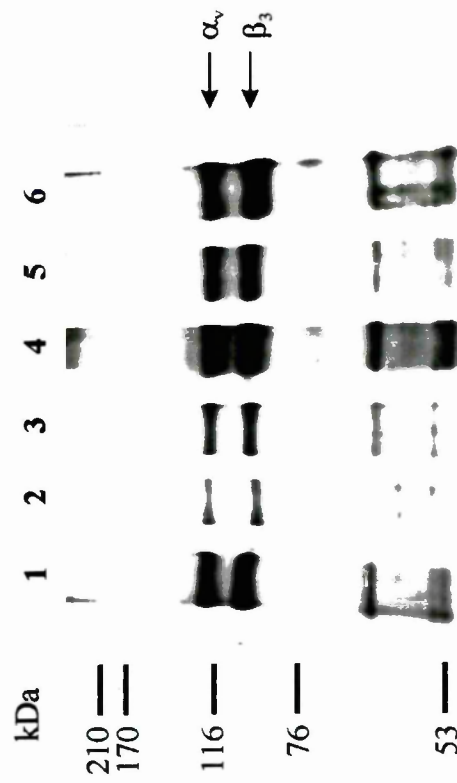


Fig. 7.8: Incubation of $\alpha_v\beta_3$ integrin with MMPs for 16 hours at 37°C. Lane 1: incubated $\alpha_v\beta_3$ integrin; 2: $\alpha_v\beta_3$ integrin + GLA (10:1); 3: $\alpha_v\beta_3$ integrin + GLB (10:1); 4: $\alpha_v\beta_3$ integrin + SL-1 (10:1); 5: $\alpha_v\beta_3$ integrin + CL-1 (10:1); 6: $\alpha_v\beta_3$ integrin + CL-3 (10:1). None of the MMPs had any significant effect on $\alpha_v\beta_3$ integrin, however, GLA activity was double-checked (see figure 7.9).

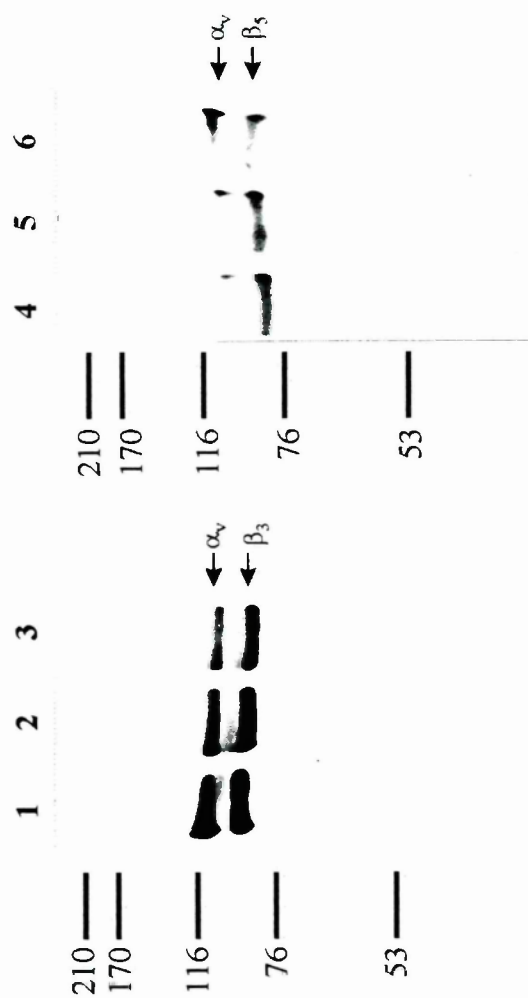


Fig. 7.9: Incubation of $\alpha_v\beta_3$ integrin and $\alpha_v\beta_5$ integrin with gelatinase A for 16 hours at 37°C. Lane 1: unincubated $\alpha_v\beta_3$ integrin; 2: incubated $\alpha_v\beta_3$ integrin; 3: $\alpha_v\beta_3$ integrin + GLA (10:1); 4: unincubated $\alpha_v\beta_5$ integrin; 5: incubated $\alpha_v\beta_5$ integrin; 6: $\alpha_v\beta_5$ integrin + GLA (10:1). GLA had no effect on either $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin.

Chapter 8

Gelatinase A Interaction With Integrin $\alpha_v\beta_3$

Introduction

Previous studies have shown that $\alpha_v\beta_3$ integrin expression and GLA production are upregulated by cells during events such as angiogenesis (Brooks et al., 1994; Cornelius et al., 1995; Friedlander et al., 1995) and tumour progression (Seftor et al., 1992; Crawford and Matrisian, 1994; Stetler-Stevenson, 1994; Danen et al., 1995a), suggesting that there may be a correlation between expression of these two proteins. Ray and Stetler-Stevenson (1995) reported that melanoma cell adhesion to vitronectin and fibronectin was significantly reduced following incubation of cells with active GLA, but did not investigate whether this was due to GLA cleavage of cell surface receptors for ECM or competitive binding of GLA to the ECM receptors. This latter possibility was substantiated by Brooks et al. (1996) and Deryugina et al. (1997) who reported that human integrin $\alpha_v\beta_3$ was able to interact directly with gelatinase A via its C-terminal domain. Brooks et al. (1996) reported co-localization of the integrin and MMP on the surface of human melanoma cells in cryostat sections, and reduction of human melanoma cell attachment to vitronectin (VN) after incubation of cells with chicken gelatinase but not by the C-terminal truncated mutant of chicken GLA. These authors also found by ELISA technique that chicken GLA or human VN bound to immobilized human $\alpha_v\beta_3$ integrin, and SDS-stable complexes of chicken GLA and human $\alpha_v\beta_3$ integrin were formed as analysed by SDS-PAGE.

It was of interest to expand these observations to include human gelatinase A interactions with human $\alpha_v\beta_3$ integrin. Although chicken GLA shares a very high sequence homology to human GLA, subtle changes in proteinase-integrin interactions might occur due to species differences. Therefore, this study aimed to mimic the

methods used by Brooks et al. (1996) using recombinant human GLA, to compare and contrast these results with the published data using chicken GLA.

Materials and Methods

Purified Integrins and MMPs

Human $\alpha_v\beta_3$ integrin purified from placenta was kindly donated by Dr. David Simmons, Oxford, UK, and Dr. Simon Goodman, Merc Laboratories, Darmstadt. Dr. Goodman also provided human $\alpha_v\beta_5$ integrin. Integrins were solublized in buffers containing 25-40 mM OG, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and stored at $-20^\circ C$. Recombinant chicken GLA in 0.2 M NaCl, 0.05 M Tris pH 7.5, 0.01 M $CaCl_2$, 0.05% Brij, 10% DMSO, and rabbit-anti-chicken GLA polyclonal antibody were kindly provided by Prof. James Quigley, School of Medicine, State University of New York, Stony Brook, NY, USA. Chicken GLA and antibody were stored at $4^\circ C$ and used immediately. 23C6 anti- $\alpha_v\beta_3$ integrin antibody was kindly provided by Prof. Michael Horton, Department of Medicine, Sir Jules Thorn Institute, UCL Medical School, The Middlesex Hospital, London, UK. Recombinant human GLA was kindly provided by Prof. Gillian Murphy, as described in Chapter 2.

Tissue Culture

A375M cells were obtained and maintained as described in Chapter 2. Cells were passaged 24-48 hours prior to experimentation and removed from the tissue culture plastic using trypsin:EDTA as described in Chapter 2. Cells were suspended in serum-free DMEM and centrifuged at 1500 g for 5-10 minutes to remove the trypsin:EDTA solution. The cell pellet was resuspended in serum free DMEM, cells counted using a haemocytometer and diluted in DMEM to the desired concentration.

Inhibition of Cell Attachment by Preincubation With GLA

This was performed essentially as described by Brooks et al. (1996). Briefly, vitronectin (1.0 µg/ml; 100µl/well) was adsorbed onto 96 well tissue culture plates (Corning) for 16 hours at 4°C. Wells were then washed with sterile PBS and blocked with 1% heat-denatured BSA in PBS for 1 hour at room temperature. Cells were removed from tissue culture flasks and incubated with and without gelatinase A in DMEM, 0.2 M MnCl₂ for 30 minutes at 37°C, prior to plating onto the washed 96 well plate. Cells were allowed to attach to the vitronectin for 15 minutes at 37°C in the presence of 0.2 mM MnCl₂. Unbound cells were washed off at the end of the assay period, and attached cells were detected as described for the cell attachment assays in Chapter 5.

Formation of SDS-stable Complexes

This was performed as described by Brooks et al. (1996). Briefly, purified integrins (0.5 µg) were incubated with APMA activated gelatinase A (see Chapter 7) in 50 mM Tris pH 7.5, 200 mM NaCl, 10 mM CaCl₂, 0.2 mM MnCl₂ (total volume of 50 µl), for 4 hours at 37°C. Reactions were stopped by addition of 4 x Laemmli sample buffer (Laemmli and Favre, 1973), (without mercaptoethanol), and analysed on 6% SDS-PAGE gels followed by silver staining. Samples were left unreduced and unboiled to maximise the stability of any protein-protein complexes during SDS-PAGE analysis. Since pro-GLA is likely to be associated with TIMP-2 *in vivo*, it was interesting to add TIMP-2 into the protein mixtures on some occasions, to study if this had any effect on complex formation.

GLA Binding to Immobilized $\alpha_v\beta_3$ Integrin

ELISA techniques were employed to determine if there was any direct interaction between isolated $\alpha_v\beta_3$ integrin and GLA. Briefly, binding of anti-chicken GLA antibody and anti-human GLA to chicken GLA was compared and the anti-chicken GLA antibody concentration optimized prior to the main assay. $\alpha_v\beta_3$ integrin (1.0 $\mu\text{g/ml}$) in TBS, 1mM CaCl_2 , 1 mM MgCl_2 , 1 mM MnCl_2 (binding buffer) was immobilised onto ELISA plates (Maxisorb, Nunc) by adsorption for 16 hours at 4°C. Unbound plastic was blocked by incubation with 50 mg/ml BSA in TBS for 1 hour at 37°C and washed off with binding buffer. Chicken GLA or human VN were plated onto the integrin or blocked plastic at 200 ng/ml for 1 hour at 37°C. Unbound ligand was flicked off and the wells washed with binding buffer prior to addition of rabbit-anti-chicken GLA or rabbit-anti-human VN (Chemicon) at 5.0 $\mu\text{g/ml}$ and 1:500 dilution respectively in binding buffer and 1.0 mg/ml BSA for 1 hour at 37°C. Unbound primary antibodies were flicked off and wells washed as before. Donkey-anti-rabbit horseradish peroxidase-conjugated secondary antibody (Jackson Labs) was added at 1:500 dilution in binding buffer and 1.0 mg/ml BSA for 1 hour at 37°C and removed as described above. Bound ligands were detected by addition of TMB reagents and the colour reaction allowed to develop. The reaction was stopped by addition of 2.5 M H_2SO_4 and the optical density of the wells analysed using a spectrophotometer set at 450 nm.

Immunolocalization of GLA and $\alpha_v\beta_3$ integrin

A375M melanoma cells were plated sparsely onto glass coverslips coated with 20 $\mu\text{g/ml}$ fibronectin and allowed to grow in DMEM in the presence of 10% FCS for 48 hours. Cells were washed with PBS and fixed for 5 minutes with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. Permeabilized cells were washed with PBS and incubated in primary antibodies (LM609 (Chemicon) or 23C6 (Prof. M. Horton) mouse-anti-human $\alpha_v\beta_3$ integrin antibodies, VB3 mouse-anti-human GLA antibody (Celltech, UK), and control mouse IgG (Sigma)) at 5.0

µg/ml in PBS with 50.0 µl/ml donkey serum (Sigma) for 30 minutes at room temperature. Cells were washed with PBS to remove the primary antibodies and incubated in 200 µl donkey-anti-mouse texas red conjugated secondary antibody (Jackson Labs) in PBS at 1:200 dilution for 30 minutes at room temperature. Cells were washed with PBS, mounted onto glass slides and sealed. Slides were stored in the dark at 4°C prior to photography.

Results

The purified human integrins were first run under reducing conditions on a 3-10% SDS gradient gel to establish their purity and confirm that they were intact (fig.8.1). Both $\alpha_v\beta_3$ integrin preparations contained protein bands of approximately 125 kDa and 115 kDa, which correspond to the reduced α_v and β_3 subunits respectively (Pytela et al., 1985). The purified $\alpha_v\beta_5$ integrin preparation contained two major bands, of approximate molecular weight of 125 kDa and 110 kDa under reducing conditions (Kim et al., 1994).

The purified integrins (0.5 µg/tube) were incubated with activated human GLA (200 ng/tube) according to the method of Brooks et al. (1996), immediately following APMA activation of the GLA (as described in Chapter 7). These authors reported a high molecular weight, SDS-stable GLA- $\alpha_v\beta_3$ integrin complex of approximately 200 kDa weight, following GLA-integrin incubation. However, after incubation of the $\alpha_v\beta_3$ integrin and $\alpha_v\beta_5$ integrin with human GLA under the conditions described in this paper, no evidence of such complexes was found using either α_v integrin. Interestingly, one $\alpha_v\beta_3$ integrin preparation also contained a significant band of protein of approximately 125 kDa. The α_v integrin is composed of two chains of 125 kDa and 25 kDa which are disulphide-bonded (Suzuki et al., 1986). The major band visualised on the gel corresponds to the larger chain of the α_v integrin subunit, suggesting that this preparation may have been less stable than the other. Major protein bands of

approximately 170 kDa, 125 kDa and 80 kDa were detected for the $\alpha_v\beta_3$ integrin-GLA mixture, and 140 kDa and 80 kDa for the $\alpha_v\beta_5$ integrin-GLA mixture (fig.8.2).

Brooks et al. (1996) reported that incubation of a hamster melanoma cell line (transfected with human β_3 integrin) with chicken gelatinase A prevented cell attachment to vitronectin. Ray and Stetler-Stevenson (1995) had previously reported that human melanoma cells attach firmly to both vitronectin and fibronectin, but that incubation of these cells with human GLA reduced cell attachment to either substrate. The observation of these authors was extended by studying the effect of incubation of A375M human melanoma cells with human GLA prior to cell attachment to FN. Since the activation state of the GLA used by Brooks et al was not obvious, GLA was added at two different concentrations in pro and active forms. The results of these experiments are summarised in table 1.

Table 1

FN conc. ($\mu\text{g/ml}$)	GLA conc.	Activation state of the GLA	Number of cells/well	Duration of assay (minutes)	Percent reduction in attachment
		APMA activated			
1.0	350 ng/ml	(APMA removed)	4×10^4	15	~16%
1.0	350 ng/ml	pro-GLA/GLA mix	4×10^4	15	~10%
1.0	350 ng/ml	pro-GLA/GLA mix	4×10^4	15	~20%
1.0	5 $\mu\text{g/ml}$	pro-GLA/GLA mix	4×10^4	15	~12%

These results show that preincubation of A375M melanoma cells with human gelatinase A had very little effect on subsequent cell attachment to a fibronectin substrate. However, the effect of GLA-incubation on melanoma cell attachment to a vitronectin substrate was not tested during this study, and remains to be elucidated.

Immunolocalization of $\alpha_v\beta_3$ integrin and GLA expression by A375M melanoma cells was performed. Cells were found to highly express $\alpha_v\beta_3$ integrin and this could be localized to focal adhesion complexes (fig.8.3, see arrow head). However, these cells produced low levels of endogenous GLA and this was always located within the golgi apparatus, never on the cell surface (fig.8.3). Control antibody showed almost no staining (fig.8.3).

Since little evidence for human GLA interaction with $\alpha_v\beta_3$ integrin could be found, a small amount of recombinant chicken GLA (as used by Brooks et al. (1996)) was obtained. This was used to check that the methods used in these experiments were correct and to confirm the data reported by Brooks et al. (1996). Analysis of isolated $\alpha_v\beta_3$ integrin interactions with chicken GLA by ELISA technique showed a very low level of GLA binding to immobilised $\alpha_v\beta_3$ integrin (fig.8.4). This was proportionally much lower than binding of VN to the same receptor but was just above the background. Insufficient chicken GLA prevented repetition of this experiment under exactly the same conditions, however, it would be necessary to repeat the experiment several times to determine if the low level of binding observed on this single occasion was consistently above background binding, and therefore significant.

Discussion

The data presented by Brooks et al. (1996) showed that chicken GLA was able to form stable interactions with purified human $\alpha_v\beta_3$ integrin, both in the isolated form and with human integrin transfected into $\alpha_v\beta_3$ integrin-negative hamster melanoma cells.

The formation of SDS-stable complexes of human GLA with $\alpha_v\beta_3$ integrin and $\alpha_v\beta_5$ integrin was tested using the conditions described by Brooks et al, but no high molecular weight complexes were observed. TIMP-2 was also included in some incubations to allow for the possibility that the chicken GLA used by Brooks et al was already complexed to TIMP-2. An example of an SDS-stable MMP-TIMP complex is reported in the literature, where Bläser et al. (1991) found that collagenase-2 was co-precipitated with TIMP-1 following Western blotting. Therefore, it was necessary to explore the possibility that the high molecular weight " $\alpha_v\beta_3$ integrin-GLA" complexes observed were in fact due to GLA-TIMP-2 complexing. However, no role for TIMP-2 was found in this study, as inclusion of TIMP-2 into the protein mixture had no effect on the formation of SDS-stable, human GLA- $\alpha_v\beta_3$ integrin complexes.

Although the melanoma cell line used by Brooks et al was not available, another human melanoma cell line, A375M, which was known to highly express $\alpha_v\beta_3$ integrin (Marshall et al., 1991) was used to test the hypothesis that preincubation of cells with GLA reduced $\alpha_v\beta_3$ integrin-mediated cell attachment to an ECM substrate. However, it was not clear from the methods of Brooks et al. (1996) whether pro or active GLA or inactive C-terminal truncated GLA (NGL) were present during the cell attachment assay or whether these were removed prior to assay. Therefore, to maximise the probability of GLA effecting A375M cell adhesion to ECM proteins, cells were allowed to attach in the presence of active and pro-MMPs. A small reduction in the percentage of cells attaching to FN after incubation with GLA compared to attachment of untreated cells was observed, but this only amounted to 10-20% of the total cell attachment. Since melanoma cell attachment to vitronectin after incubation with GLA was not tested, it is not possible to compare these results with the work of Brooks et al. (1996), although both fibronectin and vitronectin are ligands for $\alpha_v\beta_3$ integrin. Nevertheless, since GLA had very little effect on cell attachment to fibronectin, it might be suggested that GLA does not interact with the fibronectin receptor on A375M cells. A375M cells express at least three fibronectin receptors, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins, but $\alpha_v\beta_3$ has been

reported to be the more abundantly expressed of these integrins (Marshall et al., 1991). In order to speculate further on the role of GLA interactions with $\alpha_v\beta_3$ integrin during cell attachment to fibronectin, it would be necessary to determine which of the three integrin receptors mediate A375M cell binding to this substrate.

Since A375M cells express high levels of $\alpha_v\beta_3$ integrin, the difference in substrate (fibronectin rather than vitronectin) should not have had any effect on the immunolocalization of $\alpha_v\beta_3$ integrin and GLA on the surface of A375M cells. This experiment failed to demonstrate a spatial link between GLA and $\alpha_v\beta_3$ integrin, GLA was observed in the golgi apparatus whereas $\alpha_v\beta_3$ integrin was observed on the cell surface and in focal contacts. However, this experiment would have been better conducted using cells which produce high levels of GLA as A375M cells produced low levels of endogenous GLA on this occasion. Deryugina et al. (1997) also suggested that LM609 anti- $\alpha_v\beta_3$ integrin antibody may not be able to bind to $\alpha_v\beta_3$ integrin when the integrin is bound to ligands. Since a second anti- $\alpha_v\beta_3$ integrin antibody (23C6) was used alongside LM609 during the immunolocalization experiments it might be hoped that this problem had been overcome, although it is possible that 23C6 may also bind to $\alpha_v\beta_3$ integrin in a similar position to LM609.

Since no reliable demonstration that human GLA was able to interact with $\alpha_v\beta_3$ integrin was recorded during these studies, it was considered necessary to move back to recombinant chicken gelatinase A, as used by Brooks et al. (1996). A solid-phase ligand binding assay using chicken GLA and human $\alpha_v\beta_3$ integrin demonstrated a very low level of GLA binding to $\alpha_v\beta_3$ integrin above background readings. This was significantly lower than VN binding to $\alpha_v\beta_3$ integrin but of a corresponding level to that reported by Brooks et al. Given that the OD readings were so small (~0.1 unit), it is difficult to attach much relevance to these findings in a physiological context, where the ECM ligands might be expected to preferentially bind to free integrin rather than GLA, for which the integrin shows such low affinity.

The data presented here tends not to support the theory that GLA is localized to the cell surface by binding directly to $\alpha_v\beta_3$ integrin, however GLA has been shown to be activated at the cell surface by MT-1 MMP (Sato et al., 1994; Atkinson et al., 1995; Pei and Weiss, 1996). TIMP-2 is thought to interact with proGLA in order to localise proGLA in close proximity to MT-1 MMP prior to MT-1 MMP activation of GLA, thereby forming a trimolecular complex of TIMP-2, proGLA and MT-1 MMP. Although $\alpha_v\beta_3$ integrin is not thought to be involved in the GLA activation process, it is possible that once activated a cell might be able to sequester GLA at the cell surface by ligation of GLA to cell surface receptors such as integrins.

Previous studies have shown that the urokinase plasminogen activator receptor (uPAR) colocalizes with $\alpha_v\beta_5$ integrin on a human keratinocyte cell line (Reinartz et al., 1995) and with β_1 and β_3 integrins on HT1080 fibrosarcoma cells (Xue et al., 1997). Urokinase plasminogen activator (uPA) has also been colocalized with vinculin in focal adhesion plaques (Pöllänen et al., 1988), therefore suggesting that urokinase plasminogen binds to its receptor and is activated in very close proximity to this integrin. A novel family of cell surface molecules designated ADAMS which possess both an integrin binding domain and a metalloproteinase catalytic domain have been identified (Wolfsberger et al., 1995), and this raises the possibility that metalloproteinase activity may be positionally-linked to integrin function for this group of cell surface proteins.

However, apart from the work by Brooks et al, no other data concerning MMPs as novel ligands for integrins has been conclusively demonstrated. A recent paper by Deryugina et al. (1997) suggested that a glioma cell line was able to attach to the C-terminal domain of GLA and that this attachment was inhibited by function-blocking anti- $\alpha_v\beta_3$ integrin antibody. However, in this study the cells were allowed to attach for 24 hours, during which time they might be able to synthesize a provisional matrix to

which cells could attach via $\alpha_v\beta_3$ integrin, and therefore cannot be taken as direct evidence that GLA binds to $\alpha_v\beta_3$ integrin. Further studies are required before firm conclusions concerning the role of integrins as cell surface receptors for MMPs can be drawn.

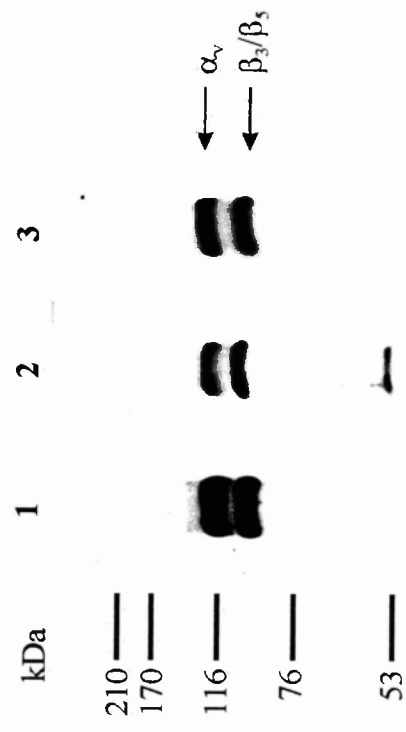


Fig.8.1: Silver stain of $\alpha_v\beta_3$ (lanes 1 and 2) and $\alpha_v\beta_5$ (lane3) integrins analysed under reducing conditions.



Fig.8.2: Silver stain of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins following incubation with GLA analysed without boiling and under nonreducing conditions. **Lane 1:** $\alpha_v\beta_3$ incubated alone; **2:** $\alpha_v\beta_3$ + GLA; **3:** $\alpha_v\beta_3$ + GLA + TIMP-2; **4:** $\alpha_v\beta_3$ + GLA; **5:** $\alpha_v\beta_3$ + GLA + TIMP-2; **6:** $\alpha_v\beta_3$ incubated alone; **7:** $\alpha_v\beta_5$ incubated alone; **8:** $\alpha_v\beta_5$ + GLA. Note that although the samples were run under nonreducing conditions, the α_v integrin subunit is composed of two chains which are linked by a disulphide bond. The integrin samples shown in lanes 3-6 appeared to be less stable than the samples in lanes 1 and 2, since the major α_v integrin band in samples 3-6 (α_v^*) was of a lower molecular weight than the major α_v integrin band of the samples in lanes 1 and 2. The reduction in α_v^* integrin mass was presumably due to loss of the lighter chain of the α_v integrin subunit in samples 3-6. No high molecular weight complexes were observed following incubation of either integrin preparation with GLA, +/- TIMP-2.

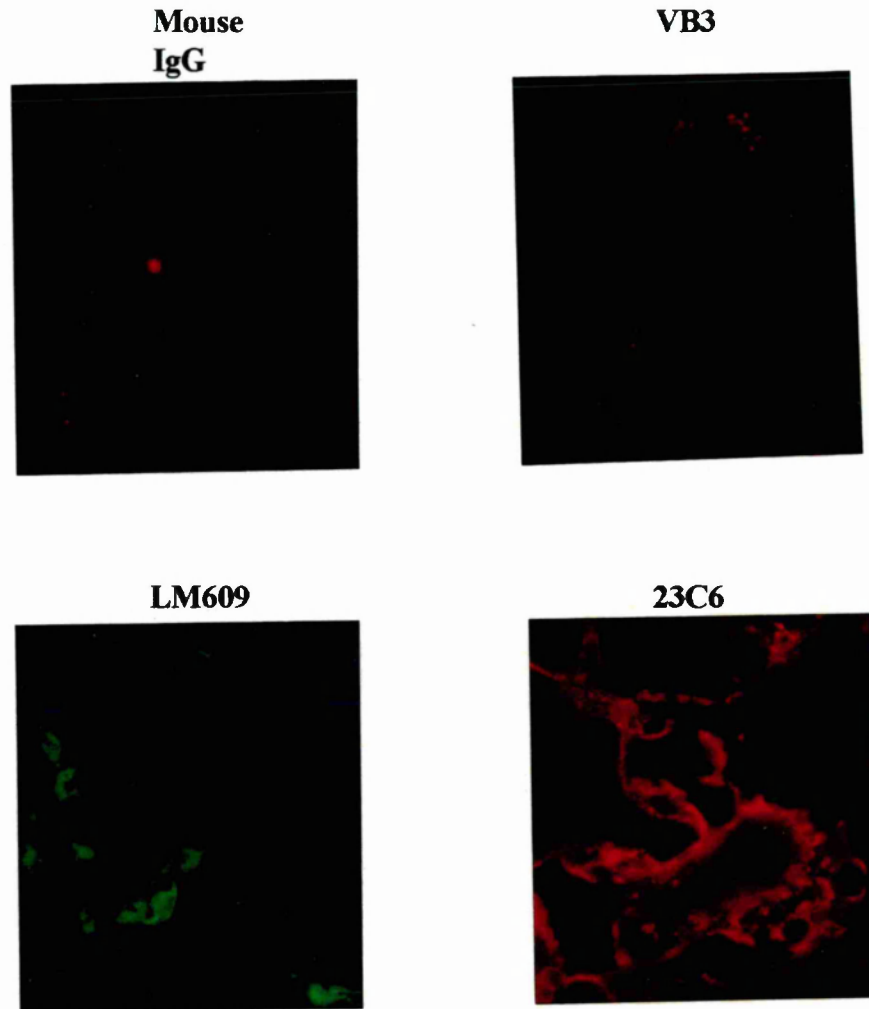


Fig.8.3: A375M cells highly expressed $\alpha_v\beta_3$ integrin but apparently did not produce much gelatinase A. Arrows indicate where $\alpha_v\beta_3$ integrin was localized in focal contacts. LM609 and 23C6 anti- $\alpha_v\beta_3$ integrin antibodies, VB3 anti-gelatinase A antibody and control mouse IgG were used at 5.0 micrograms/ml.

0 30 μ m

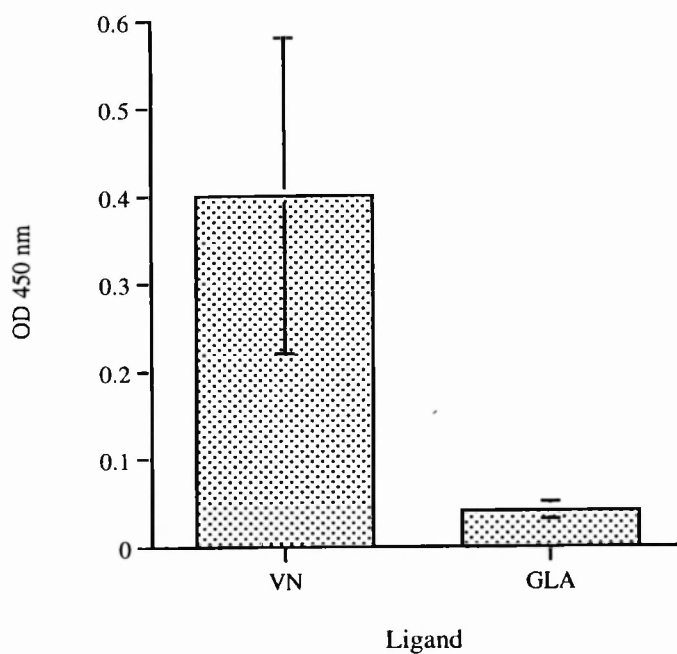


Fig.8.4 : Vitronectin (VN) and gelatinase A (GLA) binding to immobilized $\alpha_v\beta_3$ integrin. Only very low levels of GLA binding to $\alpha_v\beta_3$ integrin was observed compared to VN binding to $\alpha_v\beta_3$ integrin. Rabbit -anti-chicken GLA antibody was used at 5.0 $\mu\text{g/ml}$, rabbit-anti-human VN antibody was used at 1:500 dilution, donkey-anti-rabbit HRP-conjugated antibody was used at 1:5000 dilution.

Chapter 9

Discussion

In addition to its structural role, the ECM is an information processing medium that controls cell position, identity, proliferation and fate. This is achieved through direct cell-ECM interactions and by acting as the contextual framework for the interactions of growth factors and cytokines with their signaling receptors. Despite the profound effects the ECM can exert on cell behaviour, cells are able to modulate their responses to the ECM, even to the extent of proteolytic remodeling of the local matrix. Thus, a dynamic, bidirectional flow of information between the cells and the ECM allows individual cells to continually react and adapt to variations in their function and extracellular environment. This ability to react to change is essential for maintenance of tissue homeostasis, as all tissues constantly undergo some degree of turnover. Deregulation or blockade of this flow of information has been suggested to be a feature of pathological situations, for example tumour formation, where tumour cells lose adhesion-dependency for survival.

The results of this study clarify and advance our knowledge of $\alpha_2\beta_1$ integrin-mediated cell interactions with type I collagen following proteolytic degradation of the substrate. It was of great importance to utilize a physiological enzyme to generate type I collagen fragments, as previous studies have concentrated primarily on non-physiological cyanogen bromide-derived fragments (Staatz et al., 1990; Gulberg et al., 1992; Morton et al., 1994). Studies have mainly been aimed at elucidating the sites of integrin binding within the type I collagen molecule and have not dealt with the physiological significance of degradation of collagen *in vivo*. The approach of this study has been to present cells with native or degraded substrate in order to explore any differences in subsequent cell adhesion and migration. Such a mechanistic approach was utilized to ensure that complete degradation of type I collagen had occurred, prior to use in any of

the assays. An additional benefit of this approach was that the cleavage products of collagenase degradation of collagen could be purified, allowing initial characterization of cell interactions with the individual fragments. Type I degradation could have been achieved using cells which produce high levels of collagenolytic enzymes and which could therefore degrade the substrate during the assay periods. However, it was felt that the precleaved substrate represented a more measured route, and was more suitable for these studies.

This is thought to be a novel study of cell attachment to, and invasion through fragments of type I collagen generated by a physiological enzyme, using cells other than human platelets. Since retention of the triple-helical conformation of type I collagen CNBr fragments has been shown to be vital to the support of platelet adhesion (Morton et al., 1994), it was of great interest to study the effects of temperature on HT1080 cell interactions with the collagenase-3 fragments of type I collagen. Although the conformation of the collagen fragments generated in this study were not directly assessed, Sakai and Gross (1967) and Stark and Kühn (1968a) showed by optical rotatory dispersion that the 3/4 and 1/4 fragments of monomeric, tadpole or bacterial collagenase-treated calf skin collagen retained their triple-helical conformation below their melting temperatures (32°C and ~29°C respectively). These authors also demonstrated that both native type I collagen and collagenase-derived fragments of collagen renatured into triple-helical conformation if cooled to below melting temperature, with approximately 50% recovery of optical rotation in all cases (Stark and Kühn, 1968a). This report also showed by disc electropherogram analysis of the renatured fragments that these were mainly composed of $\alpha 1$ chains. In view of the above observations, the work of all these authors is of significance in relation to interpretation of the results of this study. Future studies using optical rotatory dispersion could confirm the triple-helical conformation of the collagen fragments used at temperatures below 29°C.

Another aspect of the experimental models used in this system is the use of thin layers of monomeric collagen. Although cells may adhere to and invade through such films, the same cells may well behave differently when cultured in thicker, 3-dimensional collagen lattices. An example of this altered response to a 3-dimensional matrix was demonstrated by Seltzer et al. (1994). These authors showed that gelatinase A produced by normal fibroblasts cultured in collagen gels was both in the pro and active forms, compared to gelatinase produced by fibroblasts cultured on collagen films, which was always in the proenzyme form. Nevertheless, collagen gels may not be the best *in vitro* model for studying the effects of an encapsulating matrix on cell behaviour.

Collagen gels consisting of polymerized monomeric collagen do not truly reflect the *in vivo* morphology of a type I collagen fibrils, which are highly cross-linked. Interestingly, an early study of the effect of chemical cross-linking agents demonstrated that cross-linking of type I collagen fibrils protected the fibrils from degradation by vertebrate collagenase, both *in vitro* and *in vivo* (Harris and Farrell, 1972). This suggests that physiological cross-linking of a mature collagenous matrix may also render it resistant to collagenase cleavage. However, during deposition of the provisional matrix produced by fibroblasts during events such as wound healing, newly formed fibrillar collagen matrices are likely to be susceptible to collagenase degradation. Type III and type I collagen are synthesized at high levels by fibroblasts relatively early on during wound healing, and are thought to be crucial for providing the healing tissue with stiffness and tensile strength. Although collagen synthesis is downregulated approximately two weeks after injury, remodelling of the provisional matrix continues for some weeks after this time (reviewed by Clark, 1996b). Thus, it is probable that a newly deposited collagen matrix is not so resistant to collagenase activity and therefore may become remodelled. This hypothesis is supported by the study of transgenic mice carrying a mutation in the type I collagen gene which prevents collagenase cleavage at the classical triple-helical site. Mice are able to develop normally due to the ability of rodent collagenase to degrade collagen at the N-telopeptide site, however in later life the

mice develop marked fibrosis of the dermis (Liu et al., 1995). This suggests that collagenase degradation of type I collagen in the triple-helical domain is necessary for normal matrix remodelling, especially in the skin.

$\alpha_2\beta_1$ integrin is one of the better characterized cell-surface ECM receptors. Its principal ligands have been determined to be type I collagen, type IV collagen (Vanderberg et al., 1991) and/or laminin-1 (Elices and Hemler, 1989; Kirchhofer et al., 1990), depending on the type of cell expressing it. Other ECM ligands such as tenascin (Sriramarao et al., 1993) and laminin-5 (A. Messent, J. Gavrilovic, P. Roussel and P. Simon-Assmann, unpublished observations) have also been suggested for $\alpha_2\beta_1$ integrin. As has already been mentioned, the type I, type II and type III collagen binding site has been determined to be in the A-domain of the α_2 integrin subunit, although it is not known if this region is involved in binding to any of the other $\alpha_2\beta_1$ integrin ligands.

As has already been mentioned in Chapter 1, generation of gelatin by heat-denaturation of type I collagen does not occur. Therefore, the only physiological mechanism for gelatinization of collagen must be by proteolytic degradation into peptides which are thermally unstable at 37°C. Using the purified $\alpha_2\beta_1$ integrin, its ligand-binding domain and collagenase-generated collagen fragments, (either melted at 37°C, or in triple-helical conformation at room temperature), this study has shown the importance of type I collagen conformation for support of $\alpha_2\beta_1$ integrin interactions.

Integrin and A-domain binding to the native 3/4 fragment of type I collagen at room temperature confirmed previous reports using non-physiological peptides, showing that $\alpha_2\beta_1$ integrin interacts with binding sites within this region of the collagen molecule. The reduction or ablation of integrin and A-domain binding to the native 1/4 fragment can be explained in at least two ways. Since some integrin but no A-domain interaction was observed at high 1/4 fragment concentrations, it may be possible that this fragment contains $\alpha_2\beta_1$ integrin binding sites of lower affinity for the integrin than the binding

sites within the 3/4 fragment, which require the whole integrin conformation to bind. Collagenase cleavage of type I collagen at the triple-helical cleavage site occurs near to the C-terminal region of the $\alpha 1(I)C N B r$ 7 peptide, and the 1/4 fragment generated by collagenase cleavage is equivalent to a small section of $\alpha 1(I)C N B r$ 7 and all of $\alpha 1(I)C N B r$ 6b (Gross et al., 1980). Morton et al. (1994) reported that CNBr 6b was unable to support human platelet adhesion, and although CNBr 7 was found to support platelet adhesion, the collagenase-generated 1/4 fragment may not contain enough of the CNBr 7 region to support α_2 integrin-mediated interactions (see fig.1.3). However, a second possibility is that the 1/4 fragment denatured during the assays at room temperature, thus ablating any integrin-mediated interactions. This would not be expected during assays performed at room temperature (23°C or below) as the published melting temperature of this fragment (~29°C; Sakai and Gross, 1967; Stark and Kühn, 1968a) is several degrees higher than the assay temperature.

The significance of these results was explored by the study of HT1080 cell interactions with the native and denatured fragments. The major integrin receptor for type I collagen expressed by these cells is $\alpha_2\beta_1$ integrin. Integrin involvement in cell attachment to collagen was confirmed using the divalent metal ion-chelator, EDTA, which removes Mg^{2+} from integrins and therefore renders cation-dependent integrins (such as $\alpha_2\beta_1$ integrin) inactive. Function-blocking anti- α_2 integrin antibodies also prevented HT1080 cell attachment to the native collagen fragments, verifying the role of $\alpha_2\beta_1$ integrin in mediating HT1080 cell attachment to the type I collagen triple helix.

Heat-destabilization by melting the collagen fragments at 37°C completely ablated HT1080 cell attachment. This result was in complete agreement to the observations made regarding isolated $\alpha_2\beta_1$ integrin and A-domain binding to the melted fragments. These cells were also unable to attach to collagen denatured at 60°C (conventional gelatin), confirming the likelihood that the collagenase fragments were in the heat-unwound, denatured conformation during these assays.

The possibility of collagenase-cleavage of type I collagen leading to the exposure of cryptic RGD sequences on the surface of the denatured fragments was investigated using A375M melanoma cells. These cells express $\alpha_v\beta_3$ integrin which, as has already been mentioned, is postulated to be a receptor for heat-denatured collagen. Since A375M cell attachment to either denatured collagen fragment could be inhibited by a function-blocking anti- $\alpha_v\beta_3$ integrin antibody, collagenase degradation of type I collagen may potentiate cell-collagen interactions via alternative integrin receptors, possibly leading to divergent signaling and modulation of cell activity. This was not possible for HT1080 cells as these cells did not apparently express $\alpha_v\beta_3$ integrin. Therefore, in the absence of any other substrate, collagenase degradation of type I collagen resulted in loss of HT1080 cell attachment. This too has profound implications for cell behaviour, as loss of appropriate cell adhesion has been shown to lead to apoptotic death of some normal cells, such as mammary epithelial cells, in a matrix-dependent manner (reviewed by Metcalfe and Streuli, 1997). Adhesion-independent survival and proliferation has also been shown to be a feature of tumour cell phenotype (reviewed by Varner and Cheresh, 1996).

A very interesting finding of this study was that collagenase degradation of type I collagen upregulated HT1080 cell motility through thin layers of collagen fragments relative to motility through native type I collagen. This was not dependent on overall protein concentration, as addition of BSA to fragment solutions to equal the concentration (w/w) of native collagen did not reduce the number of invading cells over the assay period. The accepted model of fibroblast migration involves some degree of adhesion to a substrate to allow generation of contractile forces (Palecek et al., 1997). HT1080 cells did not apparently adhere to the denatured collagen fragments. However, it may be that $\alpha_2\beta_1$ integrin undergoes sufficient transient and/or low affinity interactions with denatured collagen fragments to permit cell motility, but these interactions may not be stable enough to be detected by the attachment and binding

assays used in this study. The most definitive method for testing this hypothesis would be use of a BIAcore apparatus to study the kinetics of $\alpha_2\beta_1$ integrin interactions with native and denatured collagen fragments (discussed in more detail later in this chapter).

However, an alternative explanation for the differences in cell invasion through native and cleaved type I collagen can be suggested. The observation that HT1080 cell invasion through type I collagen fragments was upregulated relative to invasion through intact collagen, could imply that HT1080 cells plated on the collagen fragments responded differently to chemotactic ligands in the serum, compared to cells plated on native collagen. Potential ligands would include fibronectin, laminin-1 and vitronectin, which could act haptotactically, since these molecules are likely to coat the underside of the filters shortly after commencement of the assay.

Since preincubation of HT1080 cells with 6F1 anti- α_2 integrin antibody had no effect on the number of cells invading through either of the denatured collagen fragments, it is possible that this integrin may not be involved in HT1080 cell motility through native or denatured collagenous matrices. $\alpha_2\beta_1$ integrin has been clearly shown to mediate HT1080 cell attachment to type I collagen, however, it does not necessarily follow that this receptor is also responsible for regulating cell motility. This idea is supported by the findings of Liebersbach and Sanderson (1994), who showed that loss of expression of syndecan-1, a cell-surface proteoglycan receptor for type I collagen, dramatically upregulated B lymphoid cell invasion through type I collagen gels. Transfection of the syndecan-negative cells with cDNA for syndecan led to surface expression of this receptor and almost complete ablation of cell invasion. Since the level of HT1080 cell invasion through native collagen in this study was too low to investigate the role of $\alpha_2\beta_1$ integrin, future studies are required to elucidate the role of this integrin during HT1080 cell motility through native collagen. The possibility of other classes of ECM receptors mediating HT1080 cell motility through type I collagen should also be investigated.

Cells have been shown to be able to modulate expression of integrins in response to ECM and growth factor-induced signals, leading to changes in cell adhesion and motility (Chen et al., 1993; Beauvais et al., 1995; Jones et al., 1996b). Although culture of HT1080 cells on collagen-fragment coated coverslips did not apparently lead to induction of $\alpha_v\beta_3$ integrin (as determined by immunolocalization experiments), it is possible that they may utilize another integrin to interact with denatured type I collagen. As has already been mentioned, a small number of published studies suggest that $\alpha_3\beta_1$ integrin may be a collagen receptor for some cells. Yamamoto and Yamamoto (1994) reported the initial attachment of rabbit arterial smooth muscle cells to heat-denatured type I collagen was inhibited by an anti- α_3 integrin antibody. Since HT1080 cells express this integrin (Grenz et al., 1993), it is possible that these cells may upregulate $\alpha_3\beta_1$ integrin in response to the melted fragments, however this requires further study.

Interestingly, Xue et al. (1997) found that HT1080 cells expressed a low level of $\alpha_v\beta_3$ integrin when plated onto vitronectin. This integrin co-localized with uPAR in focal contacts formed on vitronectin, but the interaction was substrate-specific as uPAR co-localized principally with $\alpha_5\beta_1$ integrin when cells were plated on fibronectin. The fact that these authors could detect expression of $\alpha_v\beta_3$ integrin by HT1080 cells when others have not, suggests that expression of this integrin may be influenced by the ECM substrate with which cells are in contact. Adhesion to vitronectin may induce and/or upregulate synthesis or cell-surface expression of $\alpha_v\beta_3$ integrin, while attachment to other substrates, such as denatured type I collagen, may not induce the same upregulatory signal. HT1080 cells grown on collagen fragments did not apparently express $\alpha_v\beta_3$ integrin. It would be of interest to determine the expression of $\alpha_v\beta_3$ integrin by cells grown on vitronectin, since serum that was used as a chemoattractant in the invasion assays contains vitronectin.

Tuckwell et al. (1994) showed that HCS-2/8 human chondrosarcoma cells were able to adhere to denatured type II collagen via an $\alpha_5\beta_1$ integrin-fibronectin bridge mechanism. The possibility of HT1080 cells using such a mechanism to mediate cell invasion through type I collagen (a similar fibrillar collagen) was tested using two approaches. $\alpha_5\beta_1$ integrin is an RGD-dependent integrin and therefore a synthetic, RGD-containing peptide was added to the invasion assay system. No reduction in cell invasion was observed at the concentration of RGD peptide used in these experiments. In addition, laminin-1 as well as fibronectin was found to be a chemoattractant for HT1080 cells. Therefore, the invasion of these cells through denatured type I collagen fragments was examined using laminin-1 in place of serum. Mouse laminin-1 is known to contain an RGD site, however, since this has been shown to be cryptic (Aumailley et al., 1990) it is unlikely that cells would have interacted with laminin-1 via RGD-dependent integrins on this occasion. Since cells invaded through the collagen fragments to a similar extent when either laminin-1 or fibronectin were used as chemoattractants, it is unlikely that a fibronectin-bridge is involved in these experiments. However, it would be of interest to investigate whether laminin-1 binds to the collagenase-generated type I collagen fragments.

The results of this study demonstrating the inability of cells to adhere to collagenase-cleaved collagen fragments is very likely to be relevant to *in vivo* situations such as tumour cell migration or wound healing. Pilcher et al. (1997) demonstrated that keratinocytes are able to continue directional migration over type I collagen apparently by secreting CL-1 and degrading the collagen directly underneath each cell. The authors speculate that these cells do not attach to degraded collagen and therefore keep moving towards uncleaved collagen and chemotactic stimuli. Interestingly, keratinocytes plated on type I collagen which contains the mutation in the region of the triple-helical collagenase-cleavage site, (Liu et al., 1995), were unable to migrate compared to cells plated on normal type I collagen. This result emphasizes the major role of collagenase degradation during keratinocyte motility in this system. Collagen-binding integrin

receptors must be vital to this process of attachment and migration, and are probably involved in transducing the signal that identifies the newly-reached substrate as collagen to the migrating cell, and therefore leading to continued collagenase production and repetition of the whole process. Giannelli et al. (1997) also reported that gelatinase A cleavage of laminin-5 induced migration of breast epithelial cells. Proteolytic degradation of ECM molecules has been shown to release mitogenic and motogenic ECM-derived peptides which can influence cell behaviour (reviewed by Sage, 1997). The cellular mechanism of proteinase secretion, to induce or promote motility within an extracellular matrix environment to which a cell may possess high-affinity receptors, has potential application to the process of tumour cell invasion and metastasis (Ruoslahti, 1992; Heino, 1996).

MMPs have long been associated with the malignant phenotype, indeed, collagenase-3 was originally isolated from a breast carcinoma library (Freije et al., 1994). MMP production by tumour cells was thought necessary to clear a path for metastatic cells to move through the connective tissue stroma and intravasate the vasculature. As has already been mentioned, this simplistic hypothesis that MMPs only role in tumour cell migration was that of a proteolytic "battering ram" has since been amended, and MMPs have been shown to have many more subtle influences on tumour growth and metastasis (reviewed by Chambers and Matrisian, 1997). By producing MMPs such as collagenase-3, cells may dramatically alter their interactions with the interstitial stroma. The data presented here suggests that cleavage of type I collagen not only decreases HT1080 cell attachment at 37°C, but may also provide motogenic stimulation which can induce fibroblast chemotaxis.

A growing body of literature points to the co-ordination of integrin-mediated signals from the ECM, growth factor and cytokine stimulation and MMP production. *In vivo*, cells undergo dynamic interactions with all components of their extracellular environment, therefore it is not surprising that stimuli from various classes of cell-

surface receptor are necessary to trigger profound responses such as induction of cell motility or production of matrix-remodeling enzymes. In the experimental system used in this study, cytokines, growth factors and chemotactic proteins such as fibronectin are all present in the serum towards which the cells invade. A small percentage of these factors may well diffuse into the upper, serum-free wells in which the cells are initially placed, and thereby activate signaling pathways which co-ordinately lead to induction of migration. However, even if cells had been stimulated to move by motility factors, no movement would take place if cells were firmly attached to a substratum (as seen when HT1080 cells are plated onto native type I collagen in this system). Complex internal rearrangements such as integrin deactivation and disassembly of focal adhesions must be undergone prior to cell movement.

The signals which lead to de-adhesion and induction of motility are likely to be mediated by integrins as well as mitogens. Sundberg and Rubin (1996) reported that ligation of β_1 integrins expressed by a human diploid foreskin fibroblast cell line led to tyrosine phosphorylation of PDGF β -receptors, independent of PDGF. The authors suggest that ligand-independent PDGF β -receptor activation during cell adhesion may trigger signaling which could bring about modulation of cell interactions with the ECM. Jones et al. (1996b) showed that $\alpha_v\beta_3$ integrin receptor activation by ligand binding was necessary for smooth muscle cells to migrate over vitronectin-coated tissue culture plastic in response to IGF-1. Interestingly, these authors found that as a consequence of IGF-1 stimulation, $\alpha_v\beta_3$ integrin expression and affinity was upregulated but $\alpha_5\beta_1$ integrin expression was downregulated by these cells. The explanation offered for these observations was that $\alpha_v\beta_3$ integrin may be important for potentiating smooth muscle cell migration, and that $\alpha_5\beta_1$ integrin was either not important or inhibitory to this process.

The synergistic effects of ECM and growth factor-derived signals were emphasised by the data published by Jones et al. (1997). Tenascin-C is an ECM protein which is

produced by smooth muscle cells and acts as an autocrine survival factor for these cells when they are cultured on a floating type I collagen gel. EGF can stimulate smooth muscle cells to proliferate, but ligation of the $\alpha_v\beta_3$ integrin is necessary for EGF-dependent growth. Tenascin-C is a ligand for $\alpha_v\beta_3$ integrin, and is the likely candidate for *in vivo* stimulation of growth.

Recent reports show that integrins may physically associate with other cell surface receptors, suggesting that signals outside-in signals may be co-ordinated at the cell surface as well as downstream of the receptors. Coppolino et al. (1995b) reported that a novel isomer of the α_3 integrin subunit expressed by a prostate carcinoma cell line was covalently associated with the transferrin receptor. Since transferrin transport of iron into cells is vital for cell proliferation, $\alpha_3\beta_1$ integrin may be involved in regulation of proliferation of this type of cell.

Even more significantly, Schneller et al. (1997) reported that $\alpha_v\beta_3$ integrin physically associated with the PDGF β and insulin receptors. The mitogenic and chemotactic effects of PDGF-BB were enhanced in cells plated on a vitronectin substrate, which is a ligand for $\alpha_v\beta_3$ integrin. This again indicates that integrin activation by ligand binding leads to amplification of growth factor signals, and may represent an additional level for regulation of cell activity. To date, most mitogen and ECM-stimulated signals are thought to converge further downstream in the signaling cascade (reviewed by Dedhar and Hannigan, 1996; Giancotti, 1997). However, other growth factor receptors may well be recruited into focal adhesion complexes at the cell surface, allowing the signaling molecules of the growth factor receptor and integrin to associate and integrate.

Other cell-surface receptors such as the urokinase plasminogen activator receptor (uPAR), which binds PA and allows pericellular plasminogen activation, have been shown to interact with the leukocyte β_2 integrin subunit (Bohuslav et al, 1995). The study by Xue et al. (1997), discussed earlier, demonstrated that uPAR is also likely to

be physically associated with $\alpha_v\beta_3$ integrin and several β_1 integrins on the surface of HT1080 cells. uPAR and integrins showed maximal co-localization within focal adhesions. The integrin heterodimer which was found in association with uPAR was dependent upon the ECM substrate on which the cells were plated, on fibronectin only the α_5 integrin subunit co-localized with uPAR, on vitronectin both α_v and α_5 integrin subunits were co-localized with uPAR. However, the α_v integrin subunit was never found co-localized with uPAR on the surface of cells plated onto vitronectin, and no uPAR-integrin association at all was observed when cells were plated on polylysine-coated coverslips. This suggests that uPAR association with β_1 and β_3 integrins is both specific and ECM-regulated. Receptor co-localization was found to be inhibited by *N*-acetyl-D-glucosamine, suggesting that saccharides of the uPA and/or uPAR molecules may participate in interreceptor interactions. The functional significance of uPAR interactions with integrins is still under intense research (reviewed by Chapman, 1997), however, it is thought that uPAR forms a trimolecular complex with β integrin subunits (such as β_1 integrin) and caveolin, a membrane protein which binds signaling molecules. This complex may regulate integrin-ligand binding and integrin signaling. Cell adhesion and migration may also be affected by uPAR binding to integrins, possibly by the interaction of plasminogen activator inhibitor-1 (PAI-1) with the vitronectin-binding domain of uPAR. Integrins may also spatially regulate uPAR distribution. Hence, redistribution of the uPAR in response to ECM components could provide a mechanism to achieve polarized pericellular plasmin localization and MMP activation. In this manner, focused proteolysis of the ECM by plasmin or MMPs may lead to upregulation of tumour cell motility. Whether this method of adhesion and motility regulation has any *in vivo* significance has yet to be demonstrated. However, high levels of PAI-1 have been correlated with enhanced breast cancer metastasis, suggesting that this hypothesis requires further investigation.

A much quoted study by Brooks et al. (1996) reported that a matrix metalloproteinase, GLA, interacted directly with $\alpha_v\beta_3$ integrin on the surface angiogenic blood vessels of

chick chorioallantoic membranes and angiogenic tumour-associated blood vessels of immunodeficient mice injected with human melanoma cells. *In vitro* studies performed by this group using purified $\alpha_v\beta_3$ integrin, chicken GLA and vitronectin supported their observations *in vivo*. The authors suggest that GLA binding to $\alpha_v\beta_3$ integrin leads to cooperative protein activities which promote the invasive behaviour of cells during tissue remodeling, angiogenesis and metastasis. Deryugina et al. (1997) found that glioma cell attachment to immobilized C-terminal domain of GLA could be blocked by an anti- $\alpha_v\beta_3$ integrin antibody. These authors to suggest that $\alpha_v\beta_3$ integrin binding of GLA could localize GLA to the cell surface and focus matrix degradation, leading to focused proteolytic degradation of the pericellular matrix.

Cell-surface localization of active GLA is not a novel concept. Detection of the transmembrane MT-MMPs and the discovery that at least one of these (MT-1 MMP) can activate GLA, means that integrin binding of GLA simply to sequester it at the cell surface could be considered an example of functional redundancy. It is interesting to speculate on the physiological significance of direct binding of an active MMP to an integrin, and several major question remain to be answered. Would GLA binding to the integrin block integrin function as an ECM receptor, either by directly blocking the ligand-binding region of the integrin, or by modulating the integrin configuration by binding elsewhere on the molecule? Would binding of the C-terminal domain of GLA to the integrin interfere with TIMP inhibition of GLA activity, leading to constitutively active GLA being bound to the cell surface? Would GLA binding to the integrin interfere with integrin signaling, possibly by inhibiting integrin interactions with other cell-surface proteins or disrupting focal adhesion complex formation? Since the studies presented in this thesis fail to detect any direct interaction between $\alpha_v\beta_3$ integrin and GLA, no futher light has been shed on these questions. Therefore, important issues remain to be resolved regarding the direct interaction of GLA with $\alpha_v\beta_3$ integrin.

As previously mentioned, Jones et al. (1996b) found that ligation of $\alpha_v\beta_3$ integrin was essential for expression of tenascin-C, which acts in concert with EGF to promote smooth muscle cell survival. An interesting feature of this data was the effect of a synthetic MMP inhibitor on smooth muscle cell tenascin-C expression. When cells were cultured on native type I collagen, the inhibitor suppressed tenascin-C production. However, when cells were cultured on heat-denatured type I collagen, the MMP inhibitor had no effect on tenascin-C expression. This is most likely due to $\alpha_v\beta_3$ integrin binding to the cryptic RGD sites on type I collagen which are exposed by heat-denaturation. The authors allude to the work of Brooks et al. (1996), where GLA was postulated to bind directly to $\alpha_v\beta_3$ integrin, possibly indicating that GLA may effect tenascin-C expression by smooth muscle cells by binding to $\alpha_v\beta_3$ integrin. However, a more likely explanation which the authors themselves favour, is that MMP production by smooth muscle cells permits localized degradation of ECM components to generate RGD-containing ligands which ligate $\alpha_v\beta_3$ integrin and potentiate tenascin-C expression.

The wealth of reports showing that MMP upregulation or TIMP downregulation leads to an increase in cell invasiveness or tumourogenicity and that downregulation of MMP activity by synthetic inhibitors or TIMPs leads to a decrease in tumour cell invasiveness, strongly implies that MMPs do have a role in tumour biology. Deregulated or inappropriate MMP expression by tumour and/or stromal cells is thought to be a major contributory factor to neoplastic transformation, tumour growth and metastasis. Despite tumour cell proliferation being contact- and adhesion-independent, ECM remodeling in the vicinity of tumours probably maintains a suitable environment for sustained tumour growth. Degradation of ECM components to mitogenic peptides and release of ECM-bound growth factors are just two potential ways that MMP activity could potentiate tumour metastasis, survival and growth (Khokha et al., 1992b; Koop et al., 1994).

To enable solid tumour expansion beyond small growths, vascularization of the tumour must occur. MMPs such as GLA have been linked to angiogenesis (Gamble et al., 1993; Brooks et al., 1994; Cornelius et al., 1995) and MMP inhibitors have been shown to inhibit angiogenic blood vessel formation (Galaray et al., 1994). Vascular endothelial cell induction of GLA expression, or GLA expression by proximal stromal cells, may facilitate angiogenesis by clearing sufficient space for new vessel formation. Angiogenesis is also dependent on growth factor stimulation of vascular endothelial cells and expression of $\alpha_v\beta_3$ integrin (Kim et al., 1993; Brooks et al., 1994). One report has suggested that VEGF could induce collagenase expression by human endothelial cells (Unemori et al., 1992). Thus, MMP expression by endothelial cells may be necessary but not deregulated during angiogenesis.

Cell responses to changes in the extracellular environment can be mediated by integrins and other membrane receptors. The role of these receptors in tumour progression is still not completely understood, however, gain and/or loss of receptors for ECM components is a feature of some tumours and has been shown to modulate cell adhesion and motility (reviewed by Tang and Honn, 1994; Varner and Cheresch, 1996). Perhaps an even more significant consequence of changes in integrin receptor occupancy, is the effect that modulation of cell-ECM interactions has on integrin-mediated signaling. Since integrin signaling has been shown to affect many aspects of cellular activity, such as growth, proliferation and survival, loss of signals due to ligand degradation by MMPs is possibly more important than simple lack of adhesion to the particular degraded ECM protein.

Growth factors and cytokines have also been shown to affect MMP expression, often in association with integrin ligation. This may help to explain the observation that MMPs are often found to be produced by stromal cells which surround tumours, rather than by the tumours themselves. Examples of these phenomena is seen for collagenase-3, which has been shown to be upregulated by interleukin-6 (IL-6) in rat osteoblast

cultures (Franchimont et al., 1997), and has been shown to be regulated at the expression level by stromal-epithelial cell interactions in human breast carcinomas (Uria et al., 1997). Tumour cell proliferation is facilitated in part by tumour expression of growth factors and cytokines, which permits autocrine stimulation of growth and may influence stromal cell protein synthesis. Some studies have also identified soluble factors produced by tumour cells which stimulate stromal cells to produce MMPs and other proteins (reviewed by MacDougall and Matrisian, 1995b).

The expression of MMPs by tumour and stromal cells is now known to be under highly complex regulation which is inextricably linked to cell interactions with the ECM and cell stimulation by growth factors and cytokines. All these processes rely on information being passed down signaling cascades and inhibition of one component of the signal can have an effect on the other components. A recurrent feature of the published literature concerning the interaction of MMPs, growth factors and integrins is the feedback mechanisms by which they co-operate. This suggests that novel therapies aimed at inhibiting tumour metastasis could be targeted at more subtle aspects of tumorigenicity such as tumour cell interactions with ECM and stromal cells, rather than the obvious ones (e.g. MMP inhibition).

This thesis comprises a detailed study of $\alpha_2\beta_1$ integrin-mediated interaction with type I collagen fragments generated using a specific collagenase, that has been linked with pathological conditions such as malignant breast carcinoma. Such studies are vital to our understanding of how cells interact with damaged ECM and can give valuable insight into pathological conditions where ECM degradation plays an important part in disease progression. It is hoped further study of these interactions, particularly with reference to elucidating the mechanism by which type I collagen fragments upregulate cell motility, will contribute to a better understanding of the mechanisms by which tumour cells disseminate from the primary tumour site and metastasize to other locations within the body.

Chapter 10

Future Work

All the assays detailed in this thesis were performed using monomeric type I collagen which is soluble and can be easily adsorbed to ELISA plates or tissue culture plastic. However, *in vivo*, type I collagen assembles into insoluble fibres which cross-link to form 3-dimensional lattice structures. It is very important to bear this point in mind when extrapolating the results of the monomeric collagen studies to physiological situations. An interesting investigation by Friedl et al. (1997) studied the migration of MV3 melanoma cells in 3-dimensional collagen gels consisting of polymerized monomeric collagen. After incorporation in the lattice, MV3 cells spontaneously developed a slow migration which led to alignment of collagen fibres at attachment sites. Leading cells remodelled the collagen fibres as they moved along the collagen lattice, allowing neighbouring cells to migrate along pre-formed paths. The authors postulate the the collagen lattice remodelling may be due to both tractional forces generated by cells, which lead to mechanical bending and stretching of the matrix, and proteolytic degradation of the collagen fibres. $\alpha_2\beta_1$ integrin was found to be colocalized with cell binding sites to the collagen fibres localized at the leading edge of cells, and is probably the principal cell surface receptor which mediates MV3 cell migration through type I collagen lattices. Thus, it would be very interesting to study the effects of collagenase activity on migration of cells through type I collagen gels, possibly by utilizing a cell line which highly expresses collagenase. This could be done using time lapse video microscopy (discussed below). In addition, it would be of interest to localize the pericellular distribution of collagenase produced by cells invading through a type I collagen matrix, since proteinases have been associated with invadopodia and where their activity is thought to facilitate cell migration (Chen, 1996). This could be assessed using immunolocalization and confocal microscopy.

As mentioned briefly in Chapter 9, a very important feature of this study which remains unresolved is the molecular interaction of $\alpha_2\beta_1$ integrin with collagenase-generated type I collagen fragments in the heat-unwound (melted) conformation. A suitable apparatus for directly measuring association of these molecules is the BIAcore (Pharmacia), with which the substrate or receptor can be immobilized on a colloidal gold film, and the behaviour of the labelled receptor or substrate can be monitored very closely after addition to the film. Comparison of the kinetics of $\alpha_2\beta_1$ integrin interaction with full-length, native collagen and the melted collagen fragments would greatly facilitate interpretation of the results of the motility assays reported in this study. This type of investigation has been performed for intact laminin-1, fragments of laminin-1 and purified $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins (Pfaff et al., 1994). These authors used the BIAcore biosensor system to show that the binding affinity of the $\alpha_2\beta_1$ integrin to the P1 fragment of laminin-1 was considerably higher than the binding affinity of the $\alpha_v\beta_3$ integrin to the same laminin peptide.

Further to this point, it is necessary to analyse the expression of other ECM surface receptors (such as syndecans) in response to collagenase-cleaved collagen fragments by HT1080 cells. This type of receptor may well be very important in mediating cell motility over a substrate with which the cells have little or no interaction mediated by integrins. Syndecan-1 has been shown to be a type I collagen receptor (Koda and Bernfield, 1984) and therefore may mediate cell movement through degraded collagen, either directly or in collaboration with integrins (reviewed by Elenius and Jalkanen, 1994). Cell surface expression of syndecans could be detected by immunolocalization, or by Western blotting of cell lysates.

An important tool for comparison of cell-matrix interactions (such as native and degraded type I collagen) is time-lapse video microscopy (TVM). This allows analysis of the rate of cell movement and also provides visual information regarding cell morphology and distribution during migration. Direct comparisons could be made

between the speed of cell movement over intact type I collagen or the collagen fragments. TVM could also provide information regarding cell attachment to the melted type I collagen fragments. A particularly question which could be addressed is whether collagenase-degradation of collagen might directly lead to increased cell motility by ablation of $\alpha_2\beta_1$ integrin binding sites on type I collagen. This could be tested in the presence of chemotactic or haptotactic agents such as fibronectin or laminin-1, or alternatively, the effect of collagenase-cleavage of type I collagen on cell motility could be studied in the absence of any exogenous motility stimulant.

TVM is also an ideal medium for study of the effects of exogenous MMPs, TIMPs and synthetic metalloproteinase inhibitors on cell motility over native and degraded substrates. Cells can be monitored for several hours prior to addition of proteinases or inhibitors, and their initial and long-term response to these factors recorded and analysed. The importance of individual cell-surface ECM receptors during cell motility over native or cleaved type I collagen could also be investigated using TVM. Addition of anti-integrin antibodies or ligand-mimicking peptides into the culture system during filming may have more subtle or long-term effects on cell motility over collagen than can be observed by the invasion assay system used in this study. The role of other cell-surface collagen receptors, cell-cell adhesion receptors and growth factors during cell motility can also be examined using specific antibodies or inhibitors and TVM.

The relevance of cell-cell interactions with regard to changes in cell motility over altered matrices has not been addressed during these studies and therefore merits investigation. Tucker et al. (1990) showed that rat bladder carcinoma cells (NBT II cells) cultured on type I collagen initially migrated away from an aggregate as an epithelial sheet. Cell-cell contacts were maintained during this initial stage of migration. However, after several days in culture on type I collagen, both liver cell adhesion molecule (L-CAM) and a desmosomal protein, desmoglein, expression had dramatically dropped. This reduction in cell-cell receptors coincided with dispersal of motile cells from the original "halo" of

cells which had migrated as an epithelial sheet. Interestingly, these authors also noted that maintenance of the collagen triple-helical structure was necessary to promote cell migration. Neither heat-denatured collagen or CNBr fragments of collagen supported cell motility in this case. It would be of great interest to immunolocalize cell-cell adhesion molecules, such as the intercellular adhesion molecules (ICAMs) and desmosomal proteins, following NBT II cell culture on native and collagenase-cleaved collagen fragments.

The "outside-in" signals generated by cell interactions with native versus substrates such as type I collagen are of particular interest, since these might well influence the induction of cell motility or eventual cell death. It is possible that integrin-mediated signals transduced in response to the denatured substrate, or as a result of receptor vacancy, may well be different from the signal transduced in response to contact with intact type I collagen. This may be the case during keratinocyte migration in the provisional wound environment, where degradation of the type I collagen by collagenase-1 both facilitates and obliges cell motility over a collagen matrix (Pilcher et al., 1997).

It has been shown that the loss of integrin-mediated cell-matrix contact can induce apoptosis (specifically termed "anoikis" in this context; reviewed by Frisch et al. (1996)). It would be of great interest to explore the effects of long term culture of cells on the melted collagen fragments and compare the signals generated by this environment with those generated by culture on the native collagen molecule. If cells failed to form appropriate contact with the melted fragments during culture it is possible that normal cell activities might be prevented, possibly leading to anoikitic cell death. However, adhesion-independent cell proliferation is a feature of neoplastic transformation, and therefore it would be important to assess growth of normal and transformed cells on native collagen and the melted collagen fragments. Integrin-mediated signaling is the likely stimulus which triggers anoikis, although the signaling

pathway(s) which may be involved are not yet well understood. The role of FAK and other, downstream, cell signaling molecules such as protein kinase C (PKC) or tyrosine kinases could be investigated using reagents such as calphostin or herbimycin A, which specifically block the PKC and tyrosine kinase signaling pathways respectively. By this means it might be possible to elucidate the signaling mechanisms which are important for cell activities such as motility and/or survival.

Another aspect of cell regulation of motility and adhesion which would be interesting to explore is the effect of the expression of the null mutation of α_2 integrin in mice. Ablation of the β_1 subunit prevented the cell-surface expression of multiple integrin heterodimers, disrupting early embryonic development and leading to foetal death (Fässler and Meyer, 1995a). Most of the integrin α subunit-null mice produced to date also die during embryogenesis or shortly after birth (reviewed by Fässler et al., 1996). However, studies that have "knocked-out" the α_1 and α_3 integrin subunits have shown that the resulting mice show no phenotype after loss of the α_1 integrin (Gardner et al., 1996), and only a mild phenotype after loss of the α_3 integrin (DiPersio et al., 1997). This strongly suggests that the overlap in integrin ligand specificity can compensate for the loss of individual integrin heterodimers during development. Although the α_2 integrin subunit has yet to be knocked-out in a mouse model, a single example of a spontaneous reduction in human platelet α_2 integrin expression has been reported (Nieuwenhuis et al., 1985). This patient suffered from a bleeding disorder which was attributed to the lack of platelet interaction with type I collagen. Therefore, it might be predicted that α_2 -null mutant mice would develop and survive beyond birth. However, it would be interesting to study the effect of the mutation on events in the adult animal which are known to be partly mediated by $\alpha_2\beta_1$ integrin, such as platelet interaction with damaged vasculature.

Future studies, utilising methods such as the ones detailed above, are aimed at broadening our knowledge about the effects of MMP-degradation of type I collagen on

cell behaviour. However, these investigations could be extended to analyse the effects of MMP cleavage of other components of the provisional wound healing matrix. Particular emphasis would be placed on the effect of MMPs on cell interactions with fibrinogen, thrombospondin, fibronectin and tenascin, which are all important components of the healing wound (reviewed by Yamada and Clark, 1996). Since type IV collagen and laminin-1 are major constituents of basement membranes, it would be of great interest to investigate tumour cell interactions with the cleaved substrates, such as might be found during tumour cell metastasis. Studies are focused on acquiring a more detailed understanding of the pathways by which cells receive information and respond to dramatic changes in their microenvironment, such as proteolytic degradation of their substrate. The results of these *in vitro* studies can then be tentatively applied to the *in vivo* situation and potential therapeutic targets.

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